Dispersal Success of European Tree Frogs Thanks to Habitat Connectivity Measures: A Genetic Evaluation

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CONTENTS

GENERAL INTRODUCTION

Habitat fragmentation and connectivity projects

During the last decades, the world's natural habitats have suffered from dramatic fragmentation because of constantly growing urban sprawl and agricultural intensification. In Switzerland, the degree of spatial fragmentation is particularly alarming, since almost no other European country hosts such a high density of settlements and traffic infrastructures (Oggier et al. 2001). Furthermore, the flora and fauna of Switzerland is characterised by more threatened species and habitats than other European countries (Baur et al. 2004). Landscape fragmentation is a particular challenge to nature conservation because it confronts both animal and plant species with a whole range of problems (Fahrig 2003; Lindenmeyer & Fischer 2006). For animals, the negative consequences of habitat fragmentation often result from obstacles to movement, such as roads, which can restrict access to essential resource areas, negatively influence the probability to find mating partners, limit dispersal and migration, and prevent the colonisation of new suitable habitat patches (Trombulak & Frissell 2000; Van Dyck & Baguette 2005). Taken together, these restrictions interrupt gene flow and lead to increased genetic subdivision between populations, as well as loss of genetic diversity within populations (Strasburg 2006; Allendorf & Luikart 2007). They also increase the probability of extinction, especially in small and isolated populations, which are more susceptible to environmental and demographic stochasticity (Spielman et al. 2004; Keyghobadi 2007). In contrast, habitat connectivity is of major importance for population survival as it facilitates the dispersal of individuals and genes at the landscape scale (Crooks & Sanjayan 2006; Baguette & Van Dyck 2007). To counteract habitat fragmentation, considerable efforts have been, and continue to be, undertaken to preserve and re-establish habitat areas and to sustain or enhance connectivity among populations (Murphy & Lovett-Doust 2004; Pasqual-Hortal & Saura 2006).

In landscape management and conservation planning, appropriate habitat patches are usually first secured and their quality increased (Moilanen et al. 2005). These patches form the nodes of habitat networks, whose connectivity is subsequently enhanced by establishing movement or dispersal corridors as well as stepping-stone habitats in between the nodes (Baum et al. 2004). All of these

elements form the meshes of a habitat network and are anticipated to decrease the spatial isolation of existing populations and to provide functional connectivity among them (Beier & Noss 1998; Haddad et al. 2003; but see Levey et al. 2005). Common examples of connecting elements are wildlife passages across highways and roads such as under- and over-passes for mammals or amphibians, ecological compensation areas such as hedgerows or extensively used agricultural grasslands for mammals, birds or insects, and stepping-stone elements such as ponds or ditches for water breeding species. Concrete examples for connectivity measures in Switzerland are the prescribed ecological compensation areas on agricultural land (ÖQV; BAFU 2001) or landscape development concepts (LEKs; Bolliger et al. 2002) that integrate connectivity measures across entire landscapes, based on cantonal or regionally defined corridor areas. For instance, several cantons coordinate the securing of corridors as a default requirement for LEKs in their operative regional activities. With the same objective, specific connectivity measures are taken for species of great conservation value. This is exemplified by the conservation programs for the European tree frog in the Cantons of Argovia, Thurgau and Zurich (Rieder-Schmid 2002; Tester & Flory 2004; Meier 2004).

The European tree frog and connectivity measures

The enigmatic European tree frog (*Hyla arborea* L.) is a popular amphibian that was widespread in the Swiss lowlands before its population collapsed in the 1980s. The decline eliminated more than half of the tree frog's former distribution area in Switzerland and led to its extinction in ten cantons (Zumbach 2004). This strong decline was caused by massive destruction of its main natural breeding sites in riparian areas, but it was additionally severed by the closure and filling of gravel-pits, which provided secondary breeding habitats for tree frogs. Since then, the decline has continued as dense settlements and road systems reduce the likelihood of tree frog presence (Pellet et al. 2004). Consequently, the tree frog is listed as one of the ten endangered species from the 17 amphibian species existing in Switzerland (Schmidt & Zumbach 2005).

The European tree frog is a pioneer species and requires sunny standing water bodies that are free of fishes, contain water plants providing structure and have shallow areas to ensure warm water temperatures (Friedl & Klump 1997). Such water bodies need to be subjected to regular disturbances, as they otherwise become

quickly overgrown by vegetation and become unsuitable as tree frog breeding habitats (Tester & Flory 1995). Furthermore, such water bodies should ideally be surrounded by meadows or pastures structured by woodlots, hedgerows or forest edges, offering summer and hibernation habitats for tree frogs (Tester & Flory 1995). In such richly structured areas, tree frogs are able to move over considerable distances of one to two kilometres (Vos & Stumpel 1995). Today, exchange of individuals among neighbouring breeding sites and the colonisation of new ponds is often hindered, or completely inhibited, by highways or other roads, settlements and intensive farmland (Pellet et al. 2004). For the long-term preservation of extant tree frog populations, a functional network of interconnected breeding habitats is thus thought to be essential (Tester & Flory 1995; Glandt & Kronshage 2004).

During the 1980s, tree frogs experienced a devastating population decline in the Reuss river valley of Eastern Switzerland. To save the species from local extinction, a specific conservation project in the lower Reuss river valley of the Canton of Argovia was launched in 1991 (Tester & Flory 2004. Thereby, 90% of the remaining breeding habitats were protected and were continuously enhanced in quality according to tree frog requirements (i.e. preserving pioneer conditions; Tester & Flory 2004). Since 1993, many new stepping-stone ponds have been established to enhance the exchange of individuals among existing breeding sites and to increase overall population size (Tester & Flory 2004). In parallel, tree frog breeding sites in the adjacent upper Reuss valley of the Canton of Zurich were also protected and managed in a similar way (Cigler 1993; Cigler et al. 2002). Since 1994, all habitats occupied by tree frogs in the Reuss valley were annually monitored in both cantons by estimating male chorus sizes during breeding seasons. The habitat conservation and connectivity measures described above have impeded a further decline of the tree frog and the number of known breeding sites has since remained almost constant (Tester & Flory 2004). The total chorus size in the entire Reuss valley increased from about 500 calling males in 1994 to more than 1100 calling males in 2006 (C. Flory, CreaNatira Aargau, Ennetbaden, unpubl. data; C. Bühler, Hintermann und Weber, Basel, unpubl. data).

In contrast to the situation in the Reuss valley, many breeding sites of the European tree frog along the river Thur had already been protected during the 1980s (Beerli 1985). These protected sites were subsequently maintained according to tree frog requirements, and these measures led to stabilisation in breeding site numbers

(Kaden et al. 1995). In recent inventories conducted in the Canton Thurgau and the adjacent Canton of Zurich, the total tree frog population has been evaluated as stable or partially increasing, although new, but suitable habitats have not always been colonised (Rieder-Schmid 2002; Cigler et al. 2002). Hence, the cantonal authorities pushed connectivity measures to increase tree frog movement in the Thur valley from 2000 onwards, and a series of newly established stepping-stone ponds near Frauenfeld or in the Seebachtal valley were promptly colonised by tree frogs (Rieder-Schmid 2002). Today, the Thur valley is part of the largest continuous distribution area of the European tree frog with the highest density of occupied breeding sites in Switzerland (Zumbach 2004).

Genetic evaluation of the effectiveness in conservation management

The evaluation of the effectiveness of implemented connectivity measures is of great importance for nature conservation authorities as it facilitates future operational decision making (Beier & Noss 1998; Clevenger 2005). Such evaluations often rely on simulation studies (Falcy & Estades 2007), population mapping and population size monitoring (Maes & Bonte 2006; Petranka et al. 2007), or surveying of the direct use of connectivity elements, such as wildlife crossings, by animals (Haddad et al. 2003). One drawback of such methods is that they can not evaluate the basic goal of connectivity projects, namely the effective exchange of individuals among populations leading to functional connectivity and gene flow (Horskins et al. 2006; Strasburg 2006). However, the assessment of functional connectivity is methodologically difficult because direct long-term observations, extensive radio tracking or mark-recapture surveys of individual movement are time- and labour-intensive (Bowne & Bowers 2004). In the specific case of the European tree frog, it has been documented that populations of the Reuss and Thur valley stabilised and that newly created ponds were colonised (Cigler et al. 2002; Rieder-Schmid 2002; Tester & Flory 2004). Nevertheless, it remained unclear whether the implemented connectivity measures indeed provided functional connectivity at the landscape level and the monitoring approaches that had been applied could not evaluate whether dispersing tree frogs were effectively reproducing at new sites. This knowledge gap can be filled by the application of genetic methods to estimate the level of gene flow among populations (Mech & Hallet 2001; Dixon et al. 2006).

Population genetic approaches, such as recently developed assignment tests based on individual multilocus genotypes, are increasingly used to study contemporary or recent dispersal of individuals and gene flow (Manel et al. 2005). Assignment tests allow the detection of contemporary migration events by classifying individuals as migrants, which were born at another location than the one in which they were sampled, based on their genotype likelihood (Cornuet et al. 1999). According to the question asked and the sampling design applied, assignment methods have the potential to act as a substitute for direct-observation methods at large geographic scales (Berry et al. 2004). When populations are comprehensively sampled within a landscape, a genetic analysis is also a powerful tool to evaluate the effects of different landscape elements such as roads, forests or settlements on individual movement (Coulon et al. 2004). Hence, molecular genetic analyses are increasingly combined with landscape analyses, merging into the rapidly growing field of landscape genetics (Holderegger & Wagner 2008; Balkenhohl et al. 2009). Such applications of molecular genetic analyses are highly recommended for amphibians, since they are globally threatened through many factors driven by habitat loss and fragmentation (Cushman 2006).

Amphibians have been subjected to numerous population genetic studies (Beebee 2005). The results of these studies usually show high genetic structuring among populations (Garner et al. 2004; Arens et al. 2006; Allentoft et al. 2009; but see Brede & Beebee 2004) as well as evidence for long-term isolation, which promotes genetic load effects within populations (Rowe & Beebee 2003; Andersen et al 2004; Spear & Storfer 2008; but see Hoffman & Blouin 2004). Controversy exists on the effective dispersal ranges of amphibians, which are generally thought to be small because of the strong philopatry of many species. However, some authors argue that the mobility of amphibians, as indicated by capture and recapture experiments, is strongly underestimated (Smith & Green 2005). This stresses the importance of studying the dispersal of amphibians in a landscape genetic context with the aim of identifying realised dispersal distances and barriers to dispersal, and the exchange of reproductively successful individuals. Functional connectivity at the landscape scale can then be deduced and described.

Research questions and outline of my PhD thesis

This study evaluates the effectiveness of specific habitat connectivity measures taken for the European tree frog in Switzerland. It also establishes which landscape elements influence individual movement and genetic structure in this endangered species, as well as whether fragmentation has negative effects on the fitness of tree frog populations. The effectiveness of connectivity measures can be assessed by comparing tree frog populations in two independent landscapes that differ in population density and/or the connectivity measures that have been implemented. The Reuss and the Thur valleys meet these conditions and were selected as study sites on this basis. The breeding sites of tree frogs in the Reuss valley have a clumped distribution, with the majority of them lying in close vicinity to each other, whereas in the Thur valley, breeding sites are scattered across the landscape at larger geographical distances. Compared to the Reuss valley, specific connectivity measures were established in a later time period in the Thur valley, but tree frog breeding sites had been protected earlier than in the Reuss valley. It is therefore likely that the Thur valley reflects the natural distribution conditions of tree frogs in Switzerland. Thus, the effectiveness of connectivity measures taken in the Reuss valley can be evaluated in comparison with the situation in the Thur valley.

Chapter 1 quantifies the contemporary exchange of individuals among existing tree frog breeding sites in the Reuss and Thur valleys. When migrating individuals successfully reproduce at new sites, this exchange leaves imprints in the genome, which are detectable using high resolution molecular markers such as microsatellites (Manel et al. 2005). To highlight differences between the Reuss and Thur valleys, I first retraced the population history of the European tree frog (*Hyla arborea*) in these two landscapes. Then, I performed a comprehensive population sampling to infer the genetic structure at eleven microsatellite markers, and I used assignment tests to evaluate recent exchange of individuals among populations. I applied a non-invasive approach to genetically sample tree frogs (buccal swabs; Broquet et al. 2007).

Chapter 2 explores which landscape elements influence genetic differentiation and, hence, tree frog gene flow among breeding sites in the Reuss valley at four geographical scales. These scales were defined with respect to the probability of tree frog movement based on the results obtained in Chapter 1. I estimated the amount of gene flow from pairwise F_{ST}-values among breeding sites, and different landscape elements were assembled within corridors of 1 km width among breeding sites using

a geographic information system. In contrast to other landscape genetic studies I renounced on a pre-estimation of the negative or positive (i.e. resistance) effects of these landscape elements on gene flow. Instead, I computed multiple regression models with stepwise backward elimination and permutation testing using the F_{ST}-values as dependent and the landscape variables as well as geographic distance as independent variables (Legendre et al. 1994; Balkenhol et al. 2009).

Chapter 3 builds on the results obtained from the clustering analysis in Chapter 1 and tests whether tree frogs originating from genetically different clusters in the Reuss valley vary in fitness attributes. Since breeding sites in the Reuss valley were severely reduced during the 1980s, it is likely that isolated and genetically less diverse populations suffer from adverse effects on individual survival or fitness (Reed & Frankham 2003), as it has been detected for tree frogs in Denmark (Andersen et al. 2004) and in The Netherlands (Arens et al. 2006). I chose six breeding sites as origins for larvae, which were raised under similar conditions in a common garden experiment. Two source sites stemmed from each of three genetic clusters. Two clusters were admixed and spatially close, while the third was spatially isolated. Fitness related traits, such as survival, growth and developmental rates were tested for differences among origins using general and generalized linear models.

Chapter 4 provides an overview of my study in German and presents the main results to an audience dealing with practical nature conservation. The report highlights the most important implications of my study and states management recommendations that could be considered for both future tree frog conservation and further implementation of connectivity measures in Switzerland.

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CHAPTER 1 – Article in the Journal of Applied Ecology 2009, 46, 879-887

Population genetics suggests effectiveness of habitat connectivity measures for the European tree frog in Switzerland

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Abstract. Governmental authorities in many countries financially support the implementation of habitat connectivity measures to enhance the exchange of individuals among fragmented populations. The evaluation of the effectiveness of such measures is crucial for future management directions and can be accomplished by using genetic methods. We retraced the population history of the European tree frog in two Swiss river valleys (Reuss and Thur), performed comprehensive population sampling to infer the genetic structure at eleven microsatellite markers, and used first-generation migrant assignment tests to evaluate contemporary exchange of individuals. Compared to the Thur valley, the Reuss valley has lost almost double the number of breeding sites and exhibited a more pronounced genetic grouping. However, similar numbers of contemporary migrants were detected in both valleys. In the Reuss valley, 81 % of the migration events occurred within the identified genetic groups, whereas in the Thur valley migration patterns were diffuse. Our results show that the connectivity measures implemented in the Reuss valley facilitated effective tree frog migration among breeding sites within distances up to 4 km. Nevertheless, the Reuss valley exhibited high genetic differentiation, which reflected the impact of barriers to tree frog movement such as River Reuss. By contrast in the Thur valley, a larger number of breeding sites have been preserved and high admixture indicated exchange of individuals at distances up to 16 km. *Synthesis and applications*: We show that genetic methods can substantiate the effectiveness of connectivity measures taken in conservation management at the landscape scale. We urge responsible authorities from both river valleys to continue implementing connectivity measures and to create a dense network of breeding sites, as spatial gaps of 8 km are rarely traversed by tree frogs.

Keywords: Conservation, dispersal, first-generation migrant, fragmentation, genetic structure, genotype assignment, *Hyla arborea*, microsatellites, stepping-stone

Introduction

The continuous modification of landscapes by human activities leads to the damage and loss of natural habitats as well as to their fragmentation. Although conservation areas have been safeguarded in many countries, they often are spatially isolated remnants in otherwise intensively used landscapes. The effective isolation of such remnant habitat patches results from barriers to movement for inhabiting species and represents a particular challenge to nature conservation (Lindenmeyer & Fischer 2006). Roads, for instance, cause high mortality due to collision with vehicles (Trombulak & Frissel 2000) and such barriers not only interrupt migration, they also prevent the colonization of suitable but unoccupied habitat patches (Bowne & Bowers 2004). Habitat fragmentation may furthermore lead to increased genetic subdivision of populations, higher inbreeding and the loss of genetic diversity within populations (Allendorf & Luikart 2007; Keyghobadi 2007). Several types of measures to increase the connectivity among remnant habitats and populations are implemented by conservation managers such as protected greenways and ecological buffer zones like hedgerows or extensively used agricultural grasslands (Jongman & Pungetti 2004).

In the Swiss lowlands, urban sprawl, intensive agriculture and a dense traffic infrastructure are causing extreme habitat fragmentation (Jaeger et al. 2008). To counteract this development, Swiss federal and regional authorities financially support the implementation of habitat connectivity measures. Their aim is to enhance or re-establish the exchange of individuals among populations in fragmented landscapes. The first priority thereby is to save existing habitat patches, and secure or increase their quality (i.e. the nodes of a network; Moilanen et al. 2005), and, secondly, to set up dispersal corridors or stepping-stones between existing patches (i.e. the meshes of a network; Bennet 1999). Several species-specific projects have been initiated aimed at establishing functional connectivity among populations, including projects on the European tree frog *Hyla arborea* L. in Eastern Switzerland.

One goal of applied ecology is to evaluate the effectiveness of connectivity measures (Beier & Noss 1998). The underlying question is whether structural connectivity measures also provide functional connectivity, whereby there is effective exchange of individuals (and thereby genes) among populations (Baguette & Van Dyck 2007). However, the assessment of functional connectivity is methodologically difficult, because direct observations or mark-recapture surveys of migration are time-

and labour-intensive (Bowne & Bowers 2004). Evaluations of effectiveness thus mostly rely on monitoring trends in population size (Joseph et al. 2006) or recording the use of connectivity elements such as underpasses or wildlife crossings (Haddad et al. 2003). One drawback of these methods is that they do not evaluate the effective exchange of individuals among populations leading to gene flow (Horskins et al. 2006; Strasburg 2006). In the case of the tree frog, it is unclear whether the connectivity measures taken in Eastern Switzerland provide functional connectivity at the landscape level, despite the documentation of substantial movement distances in the species (Arens et al. 2006) and its colonisation of newly created ponds (Rieder-Schmid 2002; Tester & Flory 2004). However, contemporary or recent migration and gene flow can be studied using population genetic approaches such as assignment tests based on individual multilocus genotypes (Manel et al. 2005). Assignment methods allow the detection of contemporary migration events by classifying individuals as migrants as well as identifying their most likely population of origin (Piry et al. 2004; Paetkau et al. 2004). When sampling all or the majority of the populations within a landscape, this approach represents a powerful tool to evaluate the success of connectivity measures taken in conservation management (Berry et al. 2004; Manel et al. 2005).

Our goal was to evaluate the effectiveness of structural connectivity measures (i.e. the implementation of stepping-stone habitats) for the European tree frog in two Swiss landscapes, the Reuss and Thur valley, separated by 150 km and differing in the level of former population decline and conservation measures implemented. We retraced the population histories, inferred the genetic structure and used assignment tests to evaluate contemporary migration among populations in comprehensive samples of the two landscapes studied. Our hypothesis was that in the Reuss valley, where recent population decline was serious but many stepping-stone habitats had been established, we should find contemporary migration among populations and a genetic clustering reflecting a recent expansion out of several source areas. In contrast, in the Thur valley, where population decline was less severe and only few connectivity measures had been implemented, we expected to encounter a genetically homogenous metapopulation structure due to historical and contemporary migration.

Methods

Study species and landscapes

The European tree frog is a pioneer species that was once widespread in the Swiss lowlands before it declined to less than half of its former distribution area in the 1980s (Zumbach 2004). The decline was caused by massive habitat destruction and was additionally strengthened by the closure and infilling of gravel-pits, which are secondary breeding habitats for tree frogs. Since the 1980s, the decline has continued due to dense settlements and roads having a negative effect on tree frog presence (Pellet et al. 2004). The European tree frog is consequently listed as an endangered species in Switzerland (Schmidt & Zumbach 2005).

In the Reuss river valley in Eastern Switzerland, the tree frog has experienced such a devastating population decline that a specific conservation project was launched by Weidmann & Flory (1991). Since then the remaining breeding habitats have been protected and managed according to tree frog requirements (i.e. preserving pioneer conditions). Stepping-stone habitats have been established to provide migration routes between existing breeding sites and to increase overall population size (Tester & Flory 2004). Since 1994, all habitats occupied by tree frogs have been monitored annually, with each breeding site visited three times during the breeding season to estimate the size of male choruses. Total chorus size in the Reuss valley has increased from c. 500 calling males in 1994 to c. 1100 calling males in 2006 (Christoph Flory, ProNatura Aargau, unpubl. data; Christoph Bühler, Hintermann & Weber, unpubl. data).

In the Thur valley, the river Thur has been secured with dams resulting in the riparian area largely becoming unsuitable as a tree frog habitat. In the 1980s, many breeding sites to the north and south of the river were protected (Beerli 1985) leading to increased tree frog population sizes (Rieder-Schmid 2002; Cigler et al. 2002). Today, the Thur valley forms part of Switzerland's largest continuous area inhabited by the tree frog (Zumbach 2004). However, no monitoring programme has been put in place, and connectivity measures to increase tree frog migration were only implemented from 1999 onwards (Rieder-Schmid 2002; Cigler et al. 2002). Compared to the Reuss valley, connectivity measures were taken later, but tree frog habitats have been protected earlier.

Population history

To assess the population history of *H. arborea* in the two study landscapes, we chose three different time periods. For the Reuss valley, we took information from (1a) two inventories from 1991 and 1993 (Cigler 1993; Flory 1999), (2a) chorus size data from 1999 (Christoph Flory, ProNatura Aargau, unpubl. data; Christoph Bühler, Hintermann & Weber, unpubl. data) and (3a) chorus size data from 2006 (Christoph Flory, ProNatura Aargau, unpubl. data; Christoph Bühler, Hintermann & Weber, unpubl. data) combined with our own sampling data. For the Thur valley, we selected data from (1b) an inventory of 1994 (Kaden & Meienberger 1995), (2b) two inventories carried out in 1998 and 2002 (Rieder-Schmid 2002; Cigler et al. 2002) and (3b) our own sampling data from 2007. When calling males were present in (1a/1b), (2a/2b) and (3a/3b), a breeding site was considered as an old one. When calling males were present in (2a/2b) and/or (3a/3b), the breeding site was considered as newly colonised. When calling males were absent in (2a/2b) and (3a/3b) or (3a/3b) only, the corresponding breeding site was considered extinct. Chorus size data from 2006 and 2007 were used to assign breeding sites to two classes with <30 or >30 calling males, respectively.

Sample collection and DNA extraction

We sampled 34 of the 36 tree frog breeding sites in the Reuss valley in 2006 and 29 of the 47 sites in the Thur valley in 2007 (Fig. 1). When no males were calling at sites where tree frogs had formerly been reported, we visited the site three times before assuming the frog to be extinct at that site. Otherwise, we determined the chorus size per site, which generally agreed with numbers from recent monitoring data. We caught as many individuals as possible at sites with less than 30 calling males, and 30 individuals at sites with more than 30 calling males. Occasionally, we were able to catch females, which are distinguishable from males by the absence of vocal sacs. We took non-invasive buccal swabs from each frog for genetic analysis (Copan Italia S.p.A., Brescia, Italy; Broquet et al. 2007) and photographs from both lateral sidelines. The green back and the light-coloured belly of *H. arborea* are separated by a dark lateral stripe, which allows the identification of individuals. Buccal swabs were stored at -20 °C until DNA extraction using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) following the protocol of Broquet et al. (2007). DNA was eluted twice with 100 µL of AE buffer (QIAGEN, Hilden, Germany).

Microsatellite analysis

Ten individuals from different sites were screened for polymorphism using nine microsatellite loci from Arens et al. (2000) and eight loci from Berset-Brändli et al. (2008). Eleven primers were finally selected owing to clear patterns and consistent amplification and polymorphism in both study regions. Microsatellites were amplified using fluorescently labelled primers in four multiplex polymerase chain reactions (PCR), which were performed in 7-µL reaction volumes containing 3 µL of template DNA (10-40 ng μL^{-1}), 1× Multiplex PCR Master Mix (QIAGEN, Hilden, Germany) and 0.2 to 0.7 µM of each forward and reverse primer. Multiplex 1 consisted of primers WHA1-9 and WHA 1-103 (both 0.7 µM), multiplex 2 of primers WHA 1-104 and WHA 1-140 (both 0.7 µM), multiplex 3 of primers WHA 1-20 (0.3 µM), WHA 1-25 (0.4 µM) and WHA 1-67 (0.6 µM) and multiplex 4 of primers Ha-A127 (0.4 µM), Ha-D115 (0.6 µM), Ha-B5R3 and Ha-E2 (both 0.3 µM). Multiplex PCRs were carried out on PTC-100 Thermocyclers (MJ Research, Waltham, Massachusetts, USA) with polymerase activation at 95 °C for 15 min, followed by 30 cycles of denaturing at 95 °C for 30 s, annealing at 55 °C (multiplexes 2 and 3), 58 °C (multiplex 4) or 60 °C (multiplex 1) for 90 s and extension at 72 °C for 90 s, ending with a final extension at 72 °C for 10 min. Amplification products were run against 500 ROX™ size standard on an ABI 3130 automated sequencer (Applied Biosystems, Carlsbad, California, USA), and resulting peaks were visualized and scored using GENEMAPPER 3.7 (Applied Biosystems, Carlsbad, California, USA).

Data analysis

Multilocus genotypes were screened for repeated occurrences using the program GENALEX 6 (Peakall & Smouse 2006). Samples with matching genotypes were evaluated for repeated capture of the same individual by checking photographs of lateral stripes. In subsequent analyses, only genotype data from different individuals were used.

We tested all pairs of loci across sites within river valleys for linkage disequilibrium using the log-likelihood statistic *G* implemented in FSTAT 2.93 (Goudet 2001) and applying sequential Bonferroni correction (Rice 1989). Conformity to Hardy-Weinberg equilibrium was assessed with exact *U*-tests implemented in GENEPOP 4.0 (Raymond & Rousset 1995), which uses a Markov chain method to estimate significance.

As measures of genetic diversity, we calculated the mean number of alleles (*A*), observed heterozygosity (H_o) and expected heterozygosity (H_e) per site in GENETIX 4.03 (Belkhir et al. 1996). We used two general linear models in SPSS 10.0.1 (SPSS 2001) to test for differences in H_o and H_e between the Reuss and the Thur valley (fixed factor), with either current chorus size or *A* (as a measure of long-term population size; Allendorf & Luikart 2007) as covariates. The Spearman rank correlations between the two covariates and between the two dependent variables were determined. Since these correlations were not very high ($r_S \leq 0.644$), we retained all parameters in the models.

To estimate the effect of spatial isolation on the genetic structure of *H. arborea*, we performed isolation-by-distance tests between genetic differentiation among sites (F_{ST}/1-F_{ST}) and log-transformed geographical distances within each river valley. Mantel tests were calculated with 1000 permutations in ARLEQUIN 3.1 (Excoffier et al. 2005). We then calculated overall F_{ST}-values and their standard errors by jackknifing over loci in FSTAT 2.93 (Goudet 2001) and inferred spatial genetic structure using STRUCTURE 2.2 (Pritchard et al. 2000). For the latter, we performed ten independent runs per pre-defined cluster number (K = 1-15) using the admixture model without prior population information at burn-in lengths of 100'000 and 150'000 Markov-chain Monte Carlo sampling repeats. We determined K following the STRUCTURE manual guidelines (Pritchard et al. 2000).

In a separate analysis, we performed first-generation migrant tests in GENECLASS2 (Piry et al. 2004), to estimate contemporary migration events in the two river valleys. This test identifies migrants as individuals that were born at a breeding site other than the one in which they were sampled. Since we had sampled the majority of potential source sites, we used the ratio $L = L_{home}/L_{max}$ as the statistical criterion for the likelihood computation (L_{home} being the likelihood computed for the site where an individual was sampled and L_{max} being the highest likelihood value among all available sites including the site where the individual was sampled; Paetkau et al. 2004). We used the partially Bayesian method of Rannala & Mountain (1997) in combination with the Monte-Carlo resampling algorithm of Paetkau et al. (2004) to determine the critical value of the test statistic at α = 0.01. Finally, we calculated rough estimates of contemporary migration rates for each valley by dividing the number of individuals identified as migrants by the respective sample size.

Results

We evaluated the population history of 92 breeding sites in the Reuss valley and 74 breeding sites in the Thur valley (Fig. 1). In the Reuss valley, 25 % were old (23 sites; 21 sampled), 14 % were new (13 sites; all sampled), and 61 % of the sites were extinct (totally 56). All new sites had been created between 1993 and 2005 to act as stepping-stone habitats. They were mostly colonised by tree frogs one year after construction, with the exception of sites R33 and R34 (Table S1, Supporting Information), where tree frogs from the same region were introduced in 2000. In the Thur valley, 51 % of the sites were old (38 sites; 22 sampled), 12 % were new (9 sites; 7 sampled) and only 37 % were extinct (27 sites). Of the new sites, T4 and T15 were discovered in 2002 and T11, T12 and T17 in 2007 (Table S1, Supporting Information). Only sites T26 and T28 were created in 2000 and 2004, respectively, to act as stepping-stones. They were colonised by tree frogs during the following breeding season. Current chorus sizes with more than 30 calling males were identified in 47% and 55% of the sites in the Reuss and Thur valley, respectively.

The buccal swabbing method was efficient, as all but one sample successfully amplified in PCR. Only two pairs of samples had matching multilocus genotypes, and the comparison of lateral stripes revealed that these samples stemmed from two individuals recaptured at different breeding sites in the Reuss valley (current migration). One individual moved 0.75 km (straight-line distance) from site R10 to site R11, and the other moved 1 km from site R16 to R17. Total sampling size therefore consisted of 1169 individuals (completely genotyped at 11 loci), of which 34 were females. There was no significant linkage disequilibrium at any locus, and only sites R21 and R22 of the Reuss valley expressed significant deviations from Hardy-Weinberg equilibrium (Table S1, Supporting Information).

In both river valleys, we found high levels of neutral genetic diversity: The mean numbers of alleles (A) ranged from 1.55 to 7.64, expected heterozygosities (H_e) from 0.27 to 0.71 and observed heterozygosities (H_o) from 0.45 to 0.77 per site (Table S1, Supporting Information). Global gene diversity was lower in the Reuss than in the Thur valley (H_e = 0.618 ± 0.037 SE VS. 0.677 ± 0.053 SE). In the general linear models, log chorus size showed a significant positive relationship with H_e ($F_{1,59}$ = 147.626, $P \leq 0.001$) and H_o ($F_{1,59}$ = 4.933, P = 0.030), hence revealing a clear dependence of neutral genetic diversity from chorus size (Fig. 2). For H_e, both the effects of region and the interaction between region and log chorus size were

significant ($F_{1,59}$ = 18.448, $P \leq 0.001$ and $F_{1,59}$ = 5.357, P = 0.024, respectively). For H_o, there was neither a significant effect of region nor of the interaction. The general linear models using allele diversity as a covariate resulted in qualitatively identical results (data not shown).

Overall genetic differentiation was higher in the Reuss (F_{ST} = 0.099 ± 0.008 SE) than in the Thur valley (F_{ST} = 0.033 ± 0.004 SE), although the geographic distances among sites in the Reuss valley were generally smaller (Fig. 1). Significant isolation by distance was found in both river valleys, but it was more pronounced in the Reuss (r_m = 0.572, $P < 0.001$) than the Thur valley (r_m = 0.293, P = 0.017).

In the Reuss valley, the values of the mean logarithm of probability of the data (Ln P(X|K)) from STRUCTURE runs reached a plateau at K = 6, after which the spatial genetic clustering stabilized and the standard deviations per K started to increase (Figs. S1, S2, Supporting Information). We therefore chose K = 6 as the number of clusters best capturing the geographical clustering of the breeding sites in the Reuss valley (Fig. 3). The proportion of membership (q-mean) of sites to belong to either of these six clusters was 0.95 in cluster 1 (1 site), ranged from 0.32 to 0.78 in cluster 2 (8 sites), 0.44 to 0.81 in cluster 3 (6 sites), 0.53 to 0.85 in cluster 4 (5 sites), 0.79 to 0.97 in cluster 5 (5 sites) and from 0.87 to 0.97 in cluster 6 (9 sites). Clusters 2 and 3 showed the highest admixture since q-mean values were below 0.5 for some sites. In contrast, proportions of membership were high for clusters 1 and 6 (above 0.8), which were separated by *c.* 8 km linear distance from the populations in the centre of the Reuss valley. A substantial fraction of the two introduced sites R33 and R34 (0.85 and 0.77, respectively), located southernmost of the Reuss valley, matched with cluster 4 (Fig. 3).

In the Thur valley, a maximum of mean Ln P(X|K) was found at K = 3 and the geographical clustering pattern remained unchanged for all runs with $K > 3$ (Figs. S1, S3, Supporting Information). We therefore chose K = 3 as the number of clusters best describing the genetic grouping in the Thur valley (Fig. 3). The q-mean values of these three clusters ranged from 0.18 to 0.64 in cluster 1 (24 sites), 0.74 to 0.84 in cluster 2 (4 sites) and was 0.83 in cluster 3 (1 site). Cluster 1 covered almost the whole area of the sampled region and showed high admixture, since 20 of the 24 sites had a q-mean value below 0.5. In contrast, the proportions of memberships of clusters 2 and 3 were much higher (note that at site T20 a single individual was genotyped). Cluster 2 was located south of the river Thur in the easternmost part of

the valley. Cluster 3 consisted of site T29 only, situated southernmost of the valley and separated by about 4 km to 6 km from neighbouring sites.

The first-generation migrant tests detected 26 migrants across the Reuss valley and 24 in the Thur valley. This resulted in migration estimates of 4.5 % and 4.1 %, respectively. Unsurprisingly, most of the contemporary migration events in the Reuss valley (81 %) occurred among breeding sites within the genetic clusters identified by STRUCTURE (Figs. 3, 4) at linear distances ranging from 0.3 km to 4.0 km. Of these events, 54 % occurred among old sites, 42 % among old and new sites and only 4 % among new sites (Fig. 4). Nevertheless, five migration events occurred between different clusters: Four among clusters on the same side of the river Reuss at linear distances of 1.6 km to 4 km, and one among site R8 from cluster 2 and site R22 from cluster 5 being separated by the river Reuss at a linear distance of 3.6 km. Two of the five migration events among clusters involved new sites. There was no migration between the spatially isolated clusters 1 and 6 with any of the sites in the centre of the Reuss valley (Fig. 4). There were also no migration events between the introduced southernmost sites R33 and R34 and other sites in cluster 6.

In the Thur valley, most of the contemporary migration events (62.5 %) occurred among breeding sites within the highly admixed cluster 1. The migration events between sites occurred at linear distances ranging from 1.5 km to 16 km, also across the river Thur (Fig. 4). Sixty-two percent of migration events happened between old sites, 21 % between old and new sites and 17 % between new sites. There were no migration events detected between the breeding sites within genetic cluster 2, having a size of only 3 km. Nine migration events occurred between clusters: Eight between sites of clusters 1 and 2 at linear distances ranging from 2.25 km to 16 km (also crossing the river Thur), and one between site T22 of cluster 1 and site T29 of cluster 3, two neighbouring sites south of river Thur at a linear distance of 3.7 km. Four of the contemporary migration events between clusters involved new sites.

Discussion

We found strong differences in the level of genetic diversity and differentiation of tree frog breeding sites between the two Swiss river valleys of Reuss and Thur, although the geographical scales sampled were similar. The level of genetic variation in *H. arborea* highly depended on male chorus size, and this relationship was much more pronounced in the Reuss than the Thur valley (Fig. 2). In both landscapes, only two

sites harboured more than 100 calling males (Table S1, Supporting Information). Thus, the loss of genetic variation in small populations could generally be interpreted as a consequence of genetic drift and possibly inbreeding (Allendorf & Luikart 2007). This particularly held for the Reuss valley, where tree frogs had experienced a severe population decline. Until the early 1990s, the Reuss valley had lost 61 % of its breeding sites and today harbours fewer old breeding sites (25%) than the Thur valley (51% old sites and 37% extinct), a fact that could also explain the lower overall gene diversity in the Reuss as compared to the Thur valley (H_e = 0.618 vs. 0.677). Furthermore, the genetic subdivision of the Reuss valley was threefold (F_{ST} = 0.099 vs. 0.033), and isolation by distance was nearly twice as pronounced as in the Thur valley (r_m = 0.572 vs. 0.293). In accordance, tree frog sites in the Reuss valley exhibited substantial genetic clustering, while in the Thur valley most sites showed high admixture (Fig. 3). The higher level of genetic differentiation observed in the Reuss valley may reflect the impact of prominent barriers to movement, such as the river Reuss itself or gaps in the spatial distribution of tree frog sites (Funk et al. 2005). However, the differentiation may also have been caused by founder events with subsequent local expansion (Newman & Squire 2001).

For a scenario of genetic drift or effects of founder events in the Reuss valley, theory predicts that if isolated populations lose genetic variation due to drift, genetic distance among them should increase quickly (Hedrick 1999). If in the Reuss valley, small groups of protected breeding sites of the tree frogs became reproductively separated from each other and later on, after having recovered from the decline of the 1980s, acted as recolonization sources for newly established surrounding stepping-stone sites, this expansion on a small spatial scale will have resulted in groups of source and sink populations that were genetically similar within but different among groups (Hanski & Gagiotti 2004). Two findings that support such a recent expansion in the Reuss valley are that breeding sites were assigned to six different genetic clusters on a small spatial scale (Fig. 3) and that gene flow occurred predominantly within these clusters among both old and new sites (Fig. 4). The latter finding was also supported by the two recaptured individuals that both moved between breeding sites located within the same cluster. Nevertheless, there was evidence for some contemporary genetic exchange among clusters, but only among clusters in the central part of the Reuss valley and primarily located on the same side of the river Reuss. Gibbs (1998) suggested that rivers act as barriers to amphibian

movement. The river Reuss, 60 m wide and with a strong current, seems to represent an obstacle to tree frog migration and dispersal.

The contemporary migration events detected in the Reuss valley occurred over straight-line distances of 0.3 km to 4 km, a range that is in accordance with migration distances found in other studies on *H. arborea* (Vos et al. 2000; Arens et al. 2006) as well as reported for amphibians in general (Smith & Green 2005). As discussed above, no migration was detected between the central parts of the Reuss valley and the marginal clusters 1 and 6, indicating that a distance above 8 km is not exceeded by *H. arborea* on a regular basis, at least not in a landscape heavily disturbed by human activities. A special situation arose in the two southernmost sites R33 and R34, where tree frogs were introduced in 2000. The individuals of these two sites were assigned to cluster 4 in the central Reuss valley (Fig. 3). The most likely source sites for this introduction were sites R16 or R17, as site R18 was newly created and only colonised in 2001 (Harald Cigler, Affoltern am Albis, unpubl. data; Christoph Flory, ProNatura Aargau, unpubl. data). Although we detected no contemporary migration event among sites R32, R33 and R34, STRUCTURE analysis showed one individual at site R32 to be genetically related with the gene pool of cluster 4 (57%; Fig. 3), a result pointing to former migration events.

The Thur valley revealed a different genetic scenario. Although we found nearly as many contemporary migrant events in the Thur as in the Reuss valley, they occurred over almost the entire area sampled at distances of 1.5-16 km (Figs. 3, 4). Moreover, many migration events happened between sites situated on opposite sides of the river Thur, which is shallower river than the river Reuss and only has a width of about 30 m. This would imply that tree frogs disperse over considerable distances across the landscape and are able to cross the river Thur on a regular basis. Such regular gene exchange was also reflected in the high genetic admixture of cluster 1 (Fig. 3). Although a maximum migration distance of 12.5 km has been reported for the European tree frog, such migration distances are believed to be exceptional, especially in fragmented landscapes (Pellet et al. 2004; Arens et al. 2006). A review covering 102 studies on 53 anuran species revealed that the majority (56 %) of movement distances are below 1 km, while 44 % range from 1-10 km, and only 7% involve distances larger than 10 km, with an average movement of 2.02 km (Smith & Green 2005). In the Thur valley, this average movement distance was already exceeded by the most isolated site T29, separated from others by 3.7-6.5

km, which had received a migrant from its nearest site T22. Given these large movement distances, it is surprising that no contemporary migration was detected among the sites within cluster 2, the size of which was similar to the clusters found in the Reuss valley. All the sites within cluster 2 were constructed or reshaped during 2000 and 2004 and subsequently colonized by tree frogs (Joggi Rieder-Schmid, Kaden & Partner, unpubl. data), leading to a similar founder effect as described in the Reuss valley. The most likely explanation for the lack of detected contemporary migration events in cluster 2 is the low power of assignment tests in cases where population differentiation (F_{ST}), sample sizes and the number of loci studied are low (Manel et al. 2005). According to Cornuet et al. (1999), accurate assignment can be achieved by using at least ten microsatellite loci on 30 individuals per population with an F_{ST}-value near 0.1. In our study, these conditions were fulfilled in the Reuss valley, but not necessarily in the Thur valley and certainly not in cluster 2 with a very low F_{ST}-value of 0.019.

Our genetic analyses provide compelling evidence that the conservation and connectivity measures taken for the tree frog in the Reuss valley have been successful: Population decline has been stopped, migration among breeding sites at distances of up to 4 km is warranted, and tree frogs have expanded their range. However, the gene pools in the Reuss valley are not yet mixed, since migration among clusters is still weak and sometimes completely missing (Fig. 4). By contrast, in the Thur valley a larger number of breeding sites have been preserved, and they retained a genetic structure indicative of gene flow still contributing to the mixture of historically well connected gene pools (Fig. 3). However, one should note that it is exactly the genetic similarity among sites that causes low resolution power in assigning first-generation migrants. It would therefore be relevant to prove contemporary migration and functional connectivity in the Thur valley by either increasing the number of loci or carrying out alternative methods such as mark-recapture experiments, at least at smaller spatial scales.

We conclude that our genetic approach was successful in proving the effectiveness of connectivity measures taken in the conservation management of the European tree frog in eastern Switzerland. The genetic results not only confirm that newly established ponds are quickly colonised by tree frogs, they moreover suggest that these ponds are subsequently incorporated into a habitat network connected by considerable individual exchange. Establishing stepping-stone habitats is therefore a

successful strategy which could be adopted for other pond-breeding organisms. In doing so, attention should be given to providing high quality habitats for the target species within reachable distances and across permeable landscapes. In the case of the tree frog, distances greater than 8 km are rarely traversed by single individuals, suggesting that effective habitat networks for the species must include closely spaced refuges at 1-2 km. We therefore urge the authorities responsible for both the Thur and Reuss valleys to continue implementing connectivity measures with the long-term prospect of connecting genetic clusters. As a next step, contemporary tree frog movement should be evaluated in a landscape genetic approach (Holderegger & Wagner 2008), focussing on those landscape elements potentially forming obstacles or barriers to dispersal.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Breeding site information and genetic diversity indices.
Figure S1. Mean posterior probability plots.
Figure S2. STRUCTURE analysis for the Reuss valley.
Figure S3. STRUCTURE analysis for the Thur valley.

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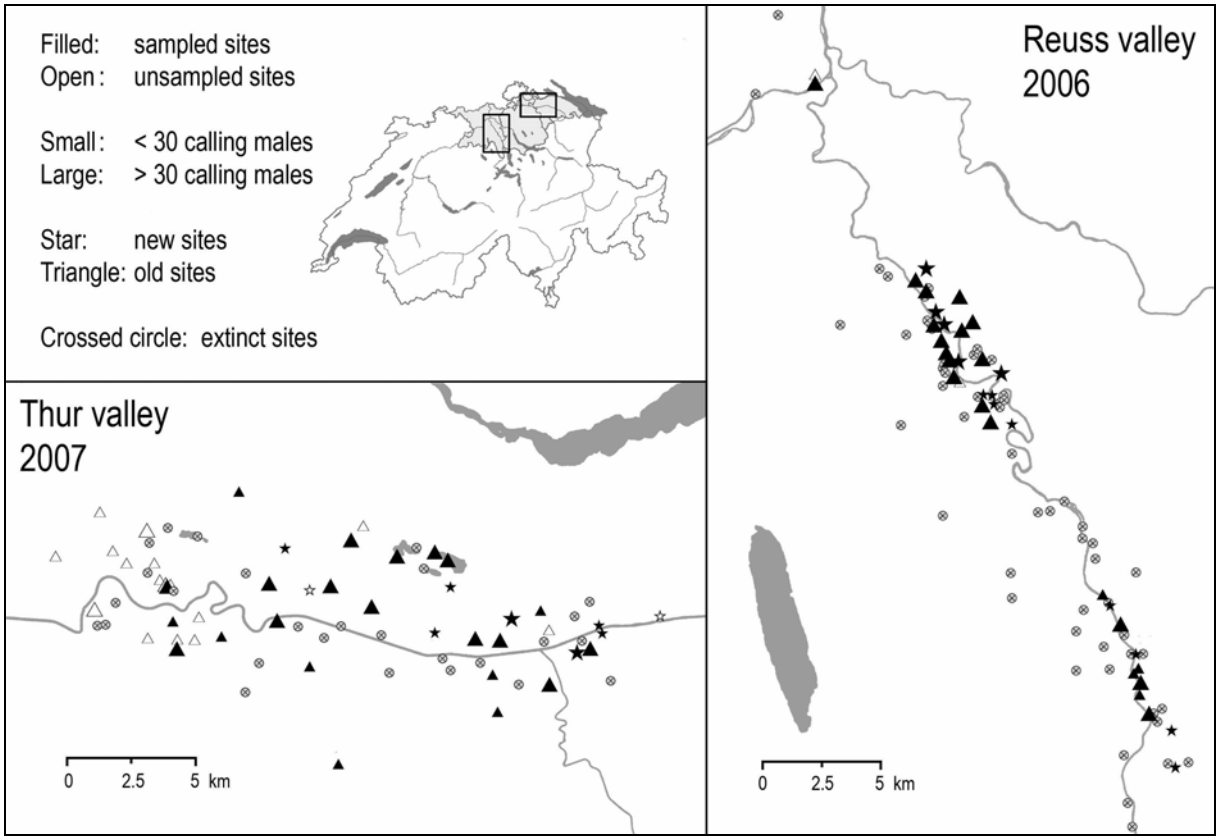

Figure 1. Summary of *Hyla arborea* population history within the Reuss and Thur valleys in Switzerland. Old sites denote breeding sites documented in 1991/3 and persisting until 2006/7. New sites denote sites originating after 1991/3 and extinct sites are sites no longer documented after 1991/3. Sampled sites were genetically analysed.

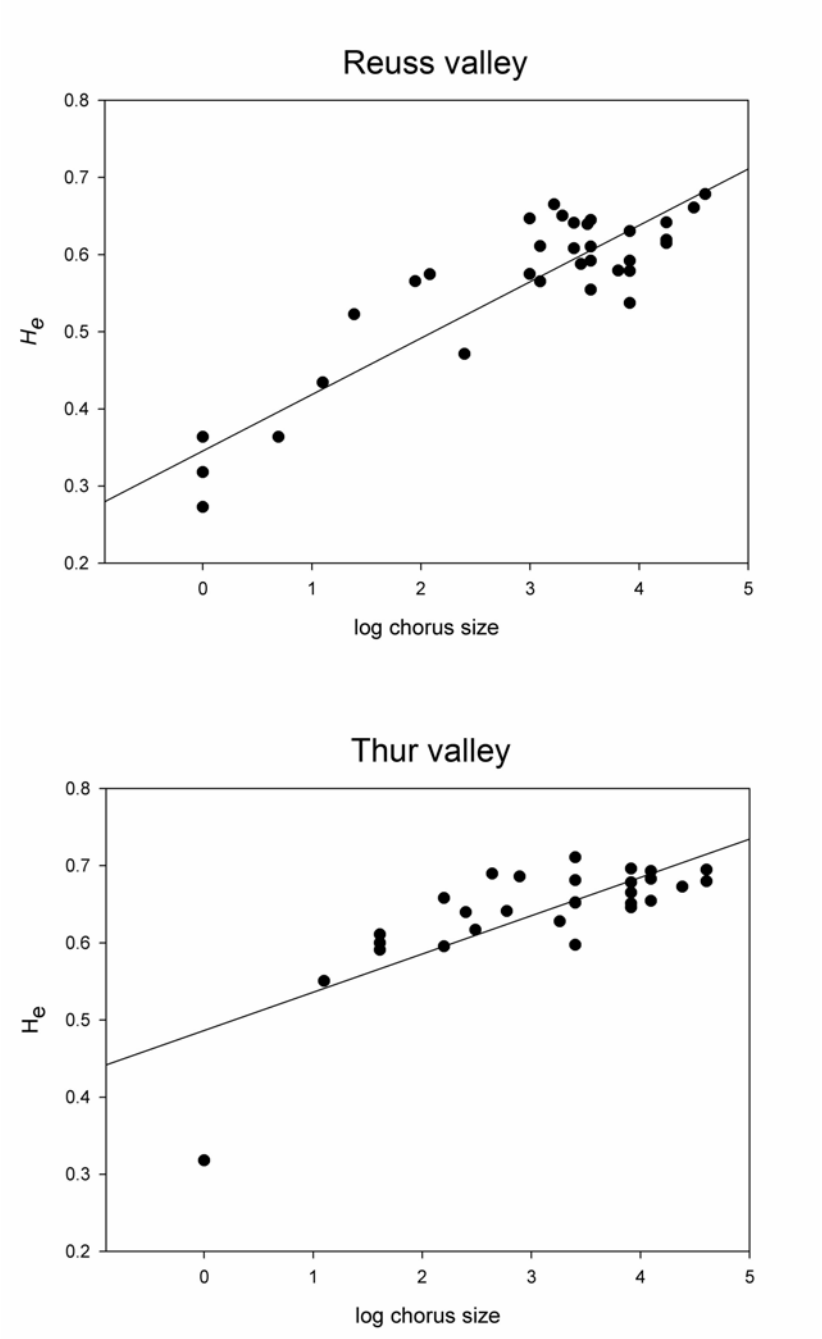

Figure 2. Scatterplots of log chorus size and expected heterozygosity (H_e) in 34 *Hyla arborea* breeding sites from the Reuss and 29 sites from the Thur valley in Switzerland.

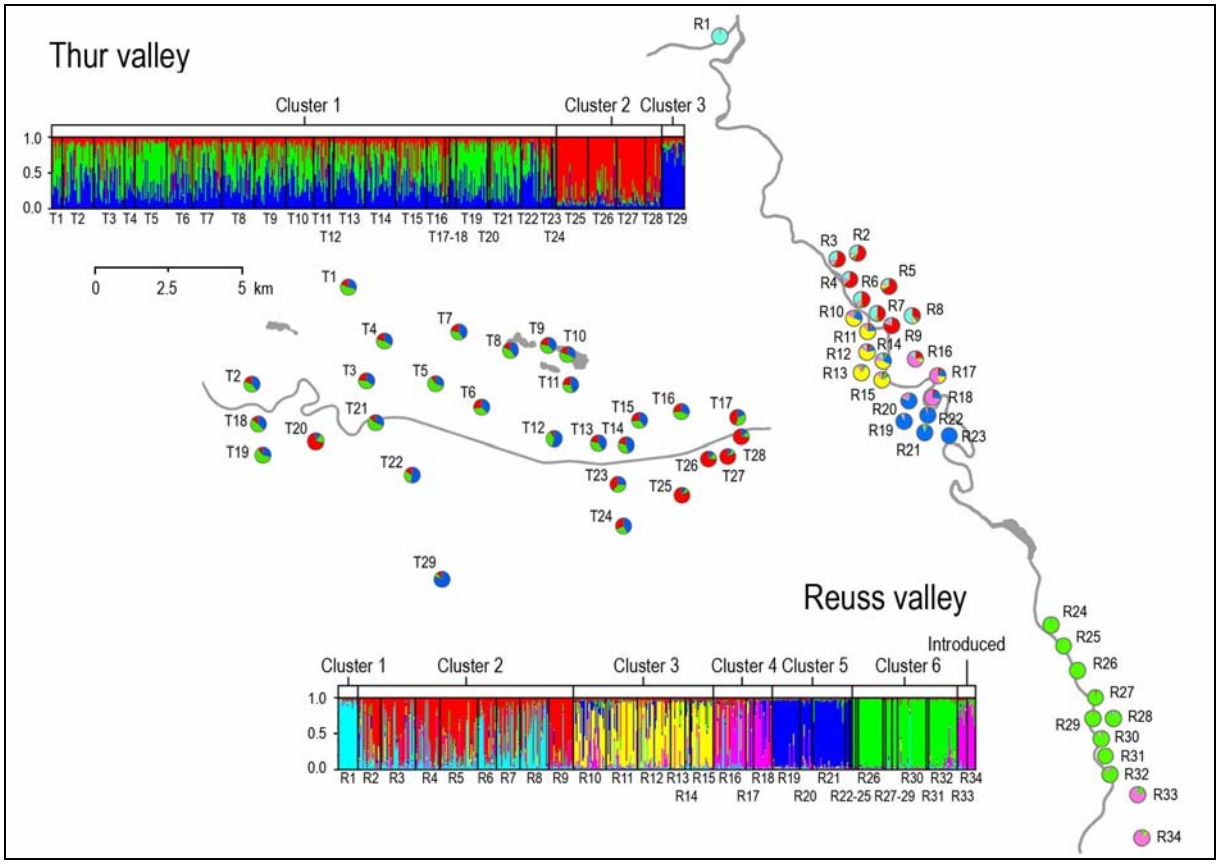

Figure 3. STRUCTURE clusters of *Hyla arborea* in the Reuss (R1-R34) and Thur (T1-T29) valley in Switzerland. The colours within bars show the proportion of membership of each individual to the genetic clusters for each valley separately. The pie charts give the genetic membership per breeding site. For site abbreviations see Table S1.

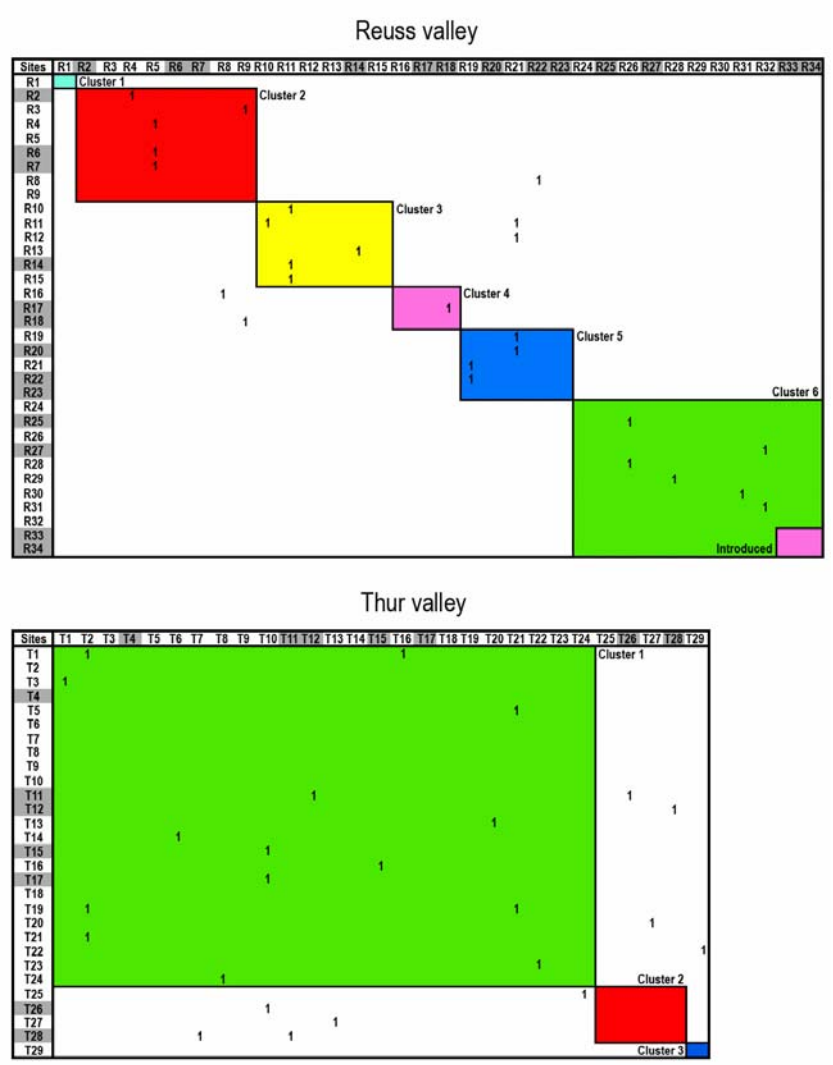

Figure 4. Migration among *Hyla arborea* breeding sites detected by first-generation migrant tests. Sites listed in columns are the immigrant sites and those in rows are the source sites. Highlighted in grey are new sites whereas the others are old. The coloured frames highlight the STRUCTURE clusters as given in Figure 3. For site abbreviations see Table S1.

Table S1. Site, abbreviation (as in Figure 3), site age, chorus and genetic sample size, mean number of alleles (*A*), and mean expected (H_e) and observed heterozygosity (H_o) with standard errors for *Hyla arborea* sites sampled in the Reuss and Thur valley in Switzerland. Significant deviations from Hardy-Weinberg equilibrium are given in bold.

Breeding site	Abbr.	Site age	Chorus size	Genetic sample size	*A*	H_e (SE)	H_o (SE)
Reuss valley							
Auschachen	R1	old	46	18	4.36	0.58 (0.27)	0.60 (0.29)
Schneeschmelze	R2	new	35	21	5.82	0.64 (0.15)	0.61 (0.21)
Aebereich	R3	old	70	33	5.73	0.64 (0.15)	0.67 (0.17)
Aegerten Stetten	R4	old	29	23	5.18	0.64 (0.15)	0.64 (0.22)
Boesimoos	R5	old	86	36	5.18	0.66 (0.11)	0.68 (0.14)
Gspiss	R6	new	30	17	4.91	0.67 (0.13)	0.71 (0.18)
Bachdole	R7	new	22	22	4.46	0.61 (0.16)	0.61 (0.18)
Honert	R8	old	35	27	4.73	0.59 (0.19)	0.58 (0.22)
Wildenau	R9	old	45	23	4.91	0.61 (0.14)	0.65 (0.20)
Klosteraecker	R10	old	50	31	5.09	0.63 (0.14)	0.65 (0.15)
Hard	R11	old	>100	30	6.55	0.68 (0.12)	0.65 (0.17)
Kraehhuebel	R12	old	72	31	5.46	0.62 (0.15)	0.62 (0.19)
Breiti	R13	old	35	13	4.73	0.64 (0.12)	0.70 (0.12)
Schlaufe	R14	new	22	4	3.64	0.57 (0.18)	0.64 (0.28)
Zelgli	R15	old	27	21	5.64	0.65 (0.11)	0.69 (0.15)
Aegerten Künten	R16	old	60	31	5.09	0.62 (0.18)	0.65 (0.21)
Raegelrain	R17	new	50	6	3.82	0.59 (0.17)	0.68 (0.23)
Foort	R18	new	20	17	4.55	0.65 (0.19)	0.69 (0.23)
Dickhoelzli	R19	old	35	26	4.18	0.55 (0.24)	0.61 (0.29)
Tote Reuss	R20	new	20	11	4.18	0.57 (0.26)	0.61 (0.27)
Schwand	R21	old	52	31	4.73	0.58 (0.23)	0.64 (0.26)
Eichholz	R22	new	3	3	2.55	0.43 (0.19)	0.48 (0.31)
Umfahrung	R23	new	1	1	1.64	0.32 (0.25)	0.64 (0.50)
Jonen Nord	R24	old	2	2	1.91	0.36 (0.25)	0.64 (0.45)
Jonen Sued	R25	new	1	1	1.55	0.27 (0.26)	0.55 (0.52)
Gmeimatt	R26	old	57	23	2.64	0.54 (0.11)	0.47 (0.20)
Muelibach	R27	new	1	1	1.73	0.36 (0.23)	0.73 (0.47)
Lunnergrien	R28	old	7	5	3.00	0.57 (0.11)	0.45 (0.25)
Leiloch	R29	old	5	4	2.64	0.52 (0.09)	0.61 (0.28)
Schlaenggen	R30	old	32	27	3.55	0.59 (0.11)	0.61 (0.08)
Lunnerallmend	R31	old	1	1	1.55	0.27 (0.26)	0.55 (0.52)
Lorzespitz	R32	old	35	27	4.18	0.61 (0.13)	0.62 (0.17)
Hinterfeld	R33	new	8	8	3.46	0.57 (0.18)	0.76 (0.30)
Grischhei	R34	new	11	7	3.27	0.47 (0.26)	0.57 (0.37)
Thur valley							
Rietbuck	T1	old	9	9	5.27	0.60 (0.24)	0.66 (0.26)
Pfaffensee	T2	old	80	30	7.27	0.67 (0.19)	0.68 (0.21)
Muelibuck	T3	old	60	29	7.27	0.69 (0.17)	0.70 (0.16)
Barchetsee	T4	new	9	9	5.73	0.66 (0.24)	0.67 (0.29)
Schulhausteich	T5	old	100	30	6.64	0.68 (0.16)	0.70 (0.16)
Chraespel	T6	old	30	24	7.45	0.68 (0.18)	0.66 (0.22)
Raffoltersee	T7	old	50	28	7.45	0.68 (0.21)	0.69 (0.21)
Im Riet	T8	old	50	31	7.64	0.70 (0.17)	0.71 (0.17)
In langen Teilen	T9	old	60	30	7.18	0.68 (0.18)	0.66 (0.21)
Buergerriet	T10	old	30	26	7.18	0.71 (0.16)	0.77 (0.17)
Privatteich Buch	T11	new	14	14	5.91	0.69 (0.15)	0.74 (0.15)
Googlete	T12	new	3	3	3.36	0.55 (0.24)	0.58 (0.34)
Chruezbuck	T13	old	60	30	7.00	0.65 (0.20)	0.67 (0.20)
Karthause Ittingen	T14	old	50	29	6.55	0.67 (0.18)	0.70 (0.18)
Grund	T15	new	30	29	7.18	0.65 (0.22)	0.65 (0.21)
Wissler	T16	old	25	17	6.55	0.64 (0.20)	0.64 (0.24)
Zielhang	T17	new	5	3	3.36	0.61 (0.10)	0.61 (0.25)
Eichholz	T18	old	5	5	3.73	0.60 (0.15)	0.60 (0.24)
Weiher Adlikon	T19	old	50	30	6.73	0.65 (0.21)	0.68 (0.20)
Guetighausen	T20	old	1	1	1.64	0.32 (0.25)	0.64 (0.50)
Aeuli	T21	old	> 100	29	6.64	**0.69 (0.19)**	**0.71 (0.21)**
Buelhuesli	T22	old	18	17	6.00	**0.69 (0.18)**	**0.66 (0.17)**
Chasperaecker	T23	old	11	11	5.09	0.64 (0.19)	0.69 (0.22)
Aegelsee	T24	old	5	3	3.55	0.59 (0.24)	0.76 (0.34)
Galgenholz	T25	old	50	30	5.18	0.65 (0.15)	0.65 (0.18)
Flutwiesen	T26	new	30	27	6.00	0.65 (0.17)	0.71 (0.22)
Altläeufe	T27	old	30	27	5.27	0.60 (0.22)	0.66 (0.26)
Gitzi	T28	new	15	15	5.55	0.62 (0.23)	0.63 (0.27)
Gruben Ebnet	T29	old	26	21	6.27	0.63 (0.21)	0.59 (0.18)

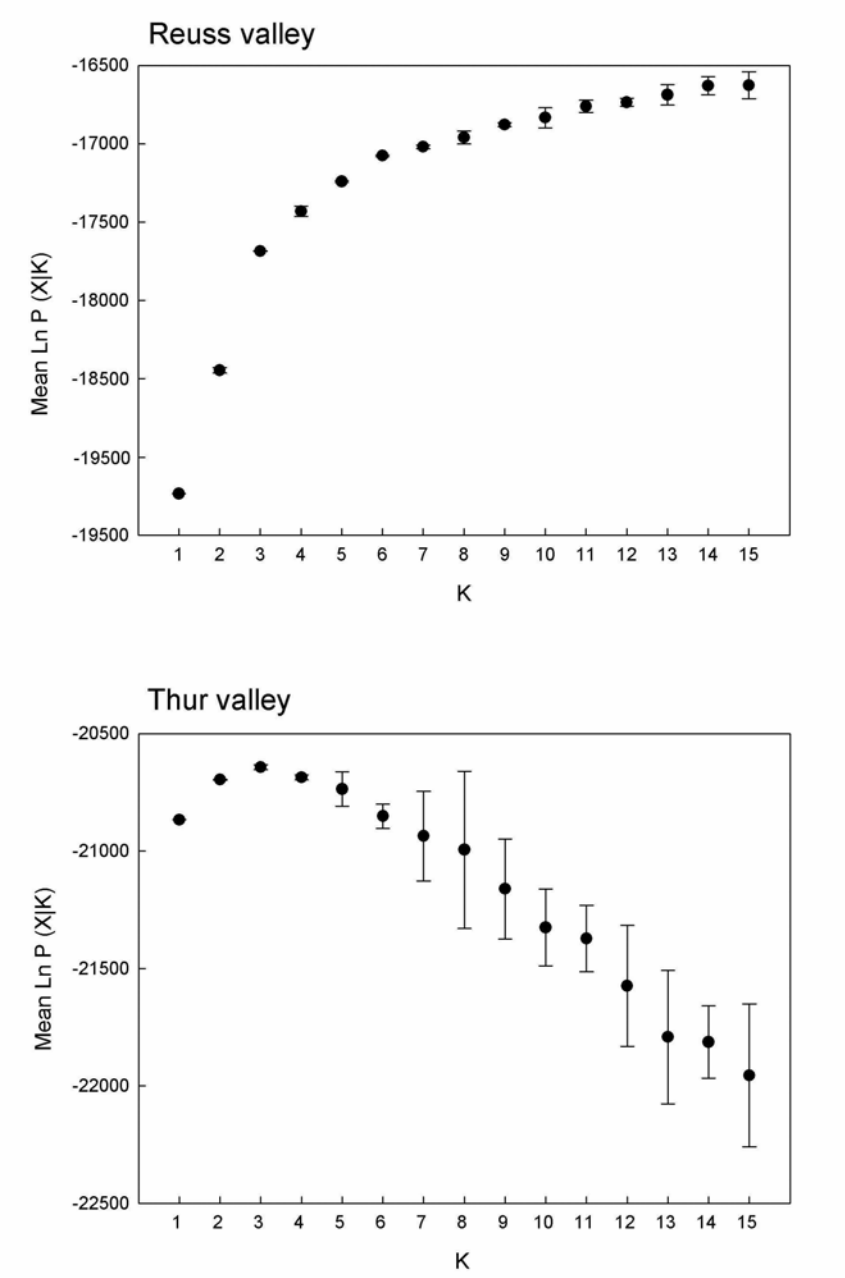

Figure S1. Plots of mean posterior probabilities (Ln P(X|K)) and standard deviations from ten independent runs in STRUCTURE calculated for *K* = 1-15 for both the Reuss and the Thur river valleys.

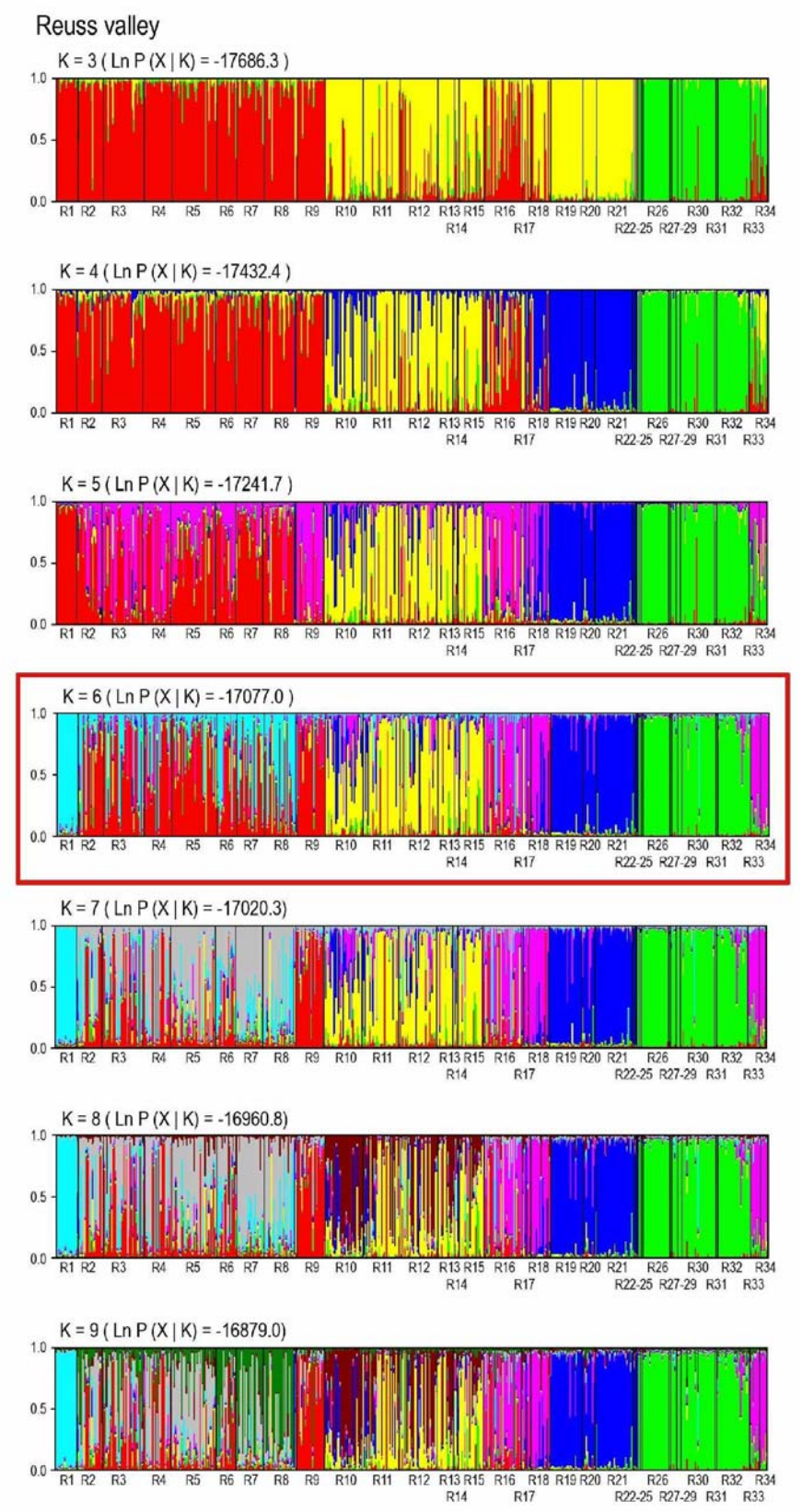

Figure S2. Proportion of membership to STRUCTURE groups (*K* = 3-9) of individuals sampled from 34 sites of *Hyla arborea* in the Swiss Reuss valley. The red frame highlights the chosen number of clusters (*K* = 6).

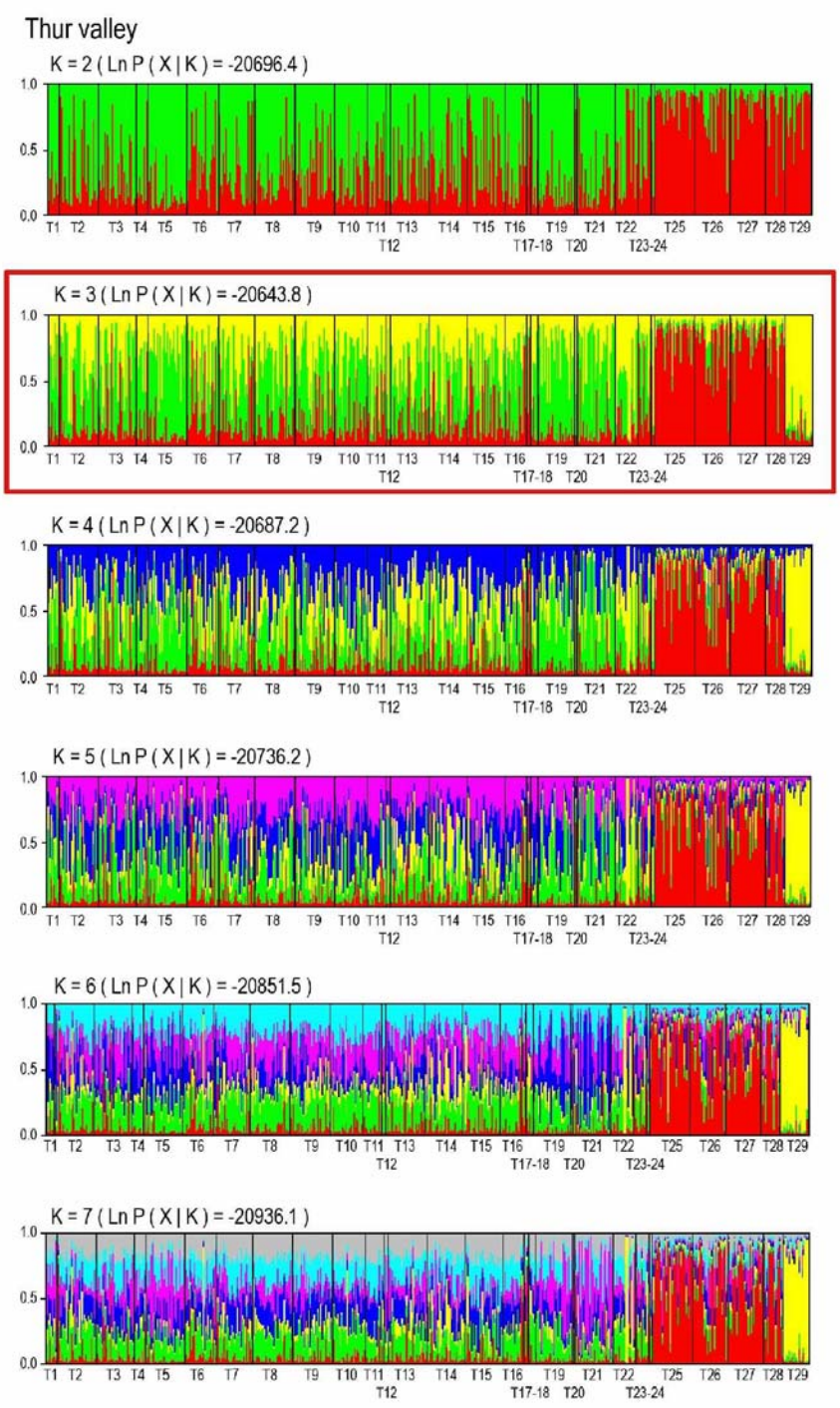

Figure S3. Proportion of membership to STRUCTURE groups (*K* = 2-7) of individuals sampled from 29 sites of *Hyla arborea* in the Swiss Thur valley. The red frame highlights the chosen number of clusters (*K* = 3).

CHAPTER 2 – Article to be submitted to Conservation Biology

Analysing where movement happens: Scale affects landscape effects on gene flow among European tree frog populations

Sonia Angelone, Felix Kienast & Rolf Holderegger

Abstract. Conservation planning often aims to restore functional connectivity between remaining populations by establishing corridors or stepping-stone elements. Connectivity greatly depends on the dispersal success of individuals, which in turn depends on landscape composition and its effect on individual movement. Fragmentation therefore strongly affects species having limited dispersal abilities such as amphibians. Here, we present a landscape genetic analysis of the European tree frog (*Hyla arborea* L.) in a fragmented landscape in the Swiss Reuss valley. We retained from predetermining resistance values of landscape elements on movement. Instead, we directly explored the effects of landscape elements and geographic distance on genetic differentiation at four distance classes reflecting different frequencies of tree frog movement. We calculated pairwise F_{ST}-values and assembled 33 landscape elements between breeding sites within corridors of 1 km width. Per distance class, we computed multiple regression models with stepwise backward elimination and permutation testing. At distances of less than 2 km between tree frog breeding sites, only the river Reuss acted as barrier to gene flow whereas surrounding tree frog breeding sites had a supportive effect. At distances between 2 km and 8 km, geographic distance had a negative effect on gene flow as well as other landscape elements such as wetlands and amphibian areas with insufficient quality for tree frogs, forests and roads. At distances greater than 8 km, the dispersal limit of tree frogs was probably reached. Our study showed that the spatial scale considered affects landscape genetic analyses, and we therefore encourage researchers to conduct their studies at spatial dimensions where migration and dispersal most likely occur.

Keywords: Conservation, dispersal distance, genetic differentiation, habitat fragmentation, *Hyla arborea*, landscape genetics, microsatellites, stepping-stone

Introduction

Habitat fragmentation and urban sprawl threaten the survival of innumerable species and have been recognized as primary causes for biodiversity loss in terrestrial ecosystems (Lindenmeyer & Fischer 2006). Remaining populations thereby become separated by human-made barriers to movement, such as highways, railways or settlements, and are forced to persist in spatially and functionally isolated habitat patches (Fahrig 2003). Even more subtle landscape changes, such as the intensification of agricultural land use, may effectively isolate populations, making them more susceptible to random demographic and genetic processes, which may negatively affect their long-term persistence (Keyghobadi 2007). However, the establishment of connectivity elements that increase individual exchange among isolated habitat patches can mitigate the negative effects of fragmentation on populations (Baguette & Van Dyck 2007): Structural connectivity may enhance or restore functional connectivity between disjunctive populations (Crooks & Sanjayan 2006). Habitat connectivity is a broadly used term in conservation planning and includes the creation of functional habitat networks by establishing corridor or stepping-stone elements among isolated patches (Jongman & Pungetti 2004).

Habitat connectivity is defined in different ways, as it depends on the interaction between the movement behaviour of a given species and the physical composition of the landscape in which movement takes place (Calabrese & Fagan 2004; Baguette & Van Dyck 2007). Structural connectivity is often measured by spatially explicit landscape metrics, which are usually quantified from the size, shape and location of habitat patches or the distances in between them (Li & Wu 2004). In contrast, functional connectivity is measured by looking at the movement of individuals either through direct observations of marked individuals (Bowne & Bowers 2004) or through indirect genetic approaches that allow the assessment of genetic differentiation or gene flow (Manel et al. 2005; Vandewoestijne & Baguette 2004). Landscape geneticists use both genetic differentiation and landscape composition approaches to evaluate those landscape elements that significantly influence gene flow (Manel et al. 2003; Holderegger & Wagner 2008). Landscape geneticists can thus determine to which extent structural elements negatively or positively affect functional connectivity within landscapes (Balkenhol et al. 2009a).

Landscape genetic approaches generally quantify the influence of landscape elements on genetic differentiation in order to identify obstacles to gene flow or to

verify the positive effects of migration corridors (Coulon et al. 2006; Cushman et al. 2006; Epps et al. 2007). Thereby, researchers often calculate several effective landscape distances and correlate them with measures of genetic differentiation of populations or genetic distances between individuals (Storfer et al. 2007; Balkenhol et al. 2009b). In such analyses, resistance values to migration are assigned to landscape elements, and the length of the path that minimizes the total resistance between two sampling locations is determined (i.e. least-cost path; Cushman et al. 2006). In corresponding studies, pure geographic straight-line distance is often used as a null model (i.e. isolation by distance; Storfer et al. 2007; Balkenhol et al. 2009b). Landscape resistance approaches, nevertheless, have some drawbacks. First, the resistance weights given to particular landscape elements often rely on expert assumptions based on the behaviour of the study organisms and not necessarily on empirical data (Storfer et al. 2007). They thus heavily rely on the experience and the prejudgement of researchers. Second, the determined least-cost path does not (necessarily) reflect the real dispersal and movement of individuals (Balkenhol et al. 2009a). Third, the partial Mantel tests usually applied in statistical analysis of such data can only evaluate the effects of one landscape element at a time (Cushman et al. 2006). Alternative analyses taking into account the effect of multiple landscape elements at a the same time without assigning them as either having a positive or negative effect on gene flow would be of high relevance to landscape genetics (Balkenhol et al. 2009b).

Generally, populations that are far from each other are only connected by exceptional long-distance dispersal, while close lying populations are interconnected by regular individual and genetic exchange (Van Dyck & Baguette 2005; Lowe 2009). The size of the study area therefore affects the quantification of landscape effects on gene flow. The impact of the spatial scale may be of particular importance in landscape genetic studies on amphibian species, since amphibians are often described as having dispersal abilities limited to a few kilometres or as exhibiting strong site fidelity (Smith & Green 2005; Cushman 2006). Moreover, many amphibian populations are threatened by habitat loss and fragmentation, and amphibian movement is thought to be negatively affected by urbanised areas or roads (Mazerolle & Desrochers 2005; Stevens & Baguette 2008). Hence, knowledge of how landscape elements affect amphibian movement or gene flow is crucial for the implementation of meaningful conservation management (Cushman 2006; Wang et

al. 2009). However, most landscape genetic studies on amphibians are performed at spatial scales largely exceeding their common movement ranges, which are expected to be well below 5 km (Smith & Green 2005; Manier & Arnold 2006; Spear & Storfer 2008). Hence, landscape variables affecting gene flow are predominantly analysed at the tail of the dispersal curve, where dispersal events are rare (Van Dyck & Baguette 2005). Moreover, by analysing minimum distances exceeding 10-20 km, many studies are analysing landscape data at a spatial scale where dispersal of amphibians hardly occurs (Telles et al. 2007; Koscinski et al. 2009). In other words, the landscape separating two sites at large distances is almost never experienced by an amphibian, as these usually move across far smaller ranges.

Here, we use an alternative approach to landscape resistance by directly analysing the effects of landscape elements on genetic differentiation among breeding sites of the European tree frog *Hyla arborea* L. without predetermining their negative or positive effects. In particular, we were interested to know which structural landscape elements influence tree frog movement at different spatial scales. Hence, we formed four distance classes based upon the knowledge gained from a genetic study on first-generation migrants in *H. arborea* in the fragmented Reuss river valley in Switzerland (Angelone & Holderegger 2009). We applied multiple regression analyses with permutation testing between pairwise genetic differentiation of *H. arborea* sites as a measurement of recurrent gene flow and various landscape variables as well as geographic distance as null expectation (i.e. isolation by distance; Wright 1943). We expected elements related to urbanization (roads and settlements; Pellet et al. 2004a) and natural elements, such as larger rivers and small lakes, to have a negative effect on, or even form barriers to, gene flow. In contrast, we expected landscape elements rich in structures, such as river edges, hedgerows and shrubs as well as wetlands, protected amphibian areas and, above all, tree frog breeding sites, to have a positive effect on gene flow in tree frogs.

Material and Methods

Study species and landscape

The European tree frog (*H. arborea* L.) is a pioneer species that experienced a drastic population decline in Switzerland because of habitat destruction and fragmentation (Angelone & Holderegger 2009). Particularly in the Reuss river valley of Northern Switzerland, half of the tree frog occurrences known in the early 1980s

had become extinct by 1991, when a specific conservation project was launched to save the remaining breeding sites (Tester & Flory 2004). Since then, habitats have been managed according to tree frog requirements (i.e. preserving pioneer conditions) in order to increase overall population size, and stepping-stone habitats have been established to enhance migration between breeding sites (Tester & Flory 2004; Angelone & Holderegger 2009). In 2006, the Reuss valley harboured approximately 1100 calling males distributed over 36 breeding sites (Angelone & Holderegger 2009; Figure1).

Genetic data

We collected genetic material by taking non-invasive buccal swabs from 582 tree frogs sampled at 34 of the 36 breeding sites in the Reuss valley during breeding season 2006 (Angelone & Holderegger 2009; Figure1). DNA was extracted using the DNeasy Tissue Kit (QIAGEN) and amplified with eleven microsatellite primers developed for *H. arborea* by Arens et al. (2000) and Berset-Brändli et al. (2008). Amplification products were run on an ABI 3130 automated sequencer (Applied Biosystems) and genotyped using GENEMAPPER 3.7 (Applied Biosystems) as reported in Angelone and Holderegger (2009). All loci and breeding sites were tested for linkage disequilibrium in FSTAT 2.93 (Goudet 2001) and for Hardy-Weinberg equilibrium in GENEPOP 4.0 (Raymond & Rousset 1995). To estimate genetic differentiation among breeding sites, we calculated pairwise F_{ST}-*values* according to Weir and Cockerham (1984) and determined their significances with 999 permutations using ARLEQUIN 3.1 (Excoffier et al. 2005).

Landscape data

We determined the coordinates of the 36 sampled breeding sites using GPS, entered them into a geographic information system and calculated geographic straight-line distances between genetically analysed breeding sites (ArcGIS 9.3; Environmental Systems Research Institute). Since tree frogs are very unlikely to overcome distances exceeding 20 km (Arens *et al.* 2006; Angelone & Holderegger 2009), we restricted landscape analyses to distances < 21 km among breeding sites, covering 96% of the full pairwise comparisons. We then assembled landscape-structural data such as forest, open land, hedgerows or rivers (Vector25, reference years 2004-2007, Swiss Federal Office of Topography), as well as data on land quality containing

information on nature protection zones, ecological compensation areas or vegetation composition (data depositories of the Federal Office of Environment and Cantonal Inventories, reference years 1995-2005). A total of 33 land characteristics was assembled and pooled into 16 landscape element classes (Appendix 1). Straight lines between breeding sites were buffered with 500 m on each side to generate a corridor of 1 km width (Figure 1). For each corridor, we calculated the proportion of area of each of the 16 landscape classes and the density of the corresponding patches. Since we were interested in detecting landscape elements that significantly influenced gene flow among tree frog breeding sites, we renounced on predetermining the positive or negative effect of the 16 landscape classes on tree frog movement. Instead, we directly used the proportion and density data within the 1 km corridors (i.e. a pairwise data set comprising 32 landscape variables) together with geographic straight-line distances between breeding sites in statistical analyses.

Data analysis

In Angelone and Holderegger (2009), first-generation migrant assignment tests revealed 26 migration events among breeding sites at distances ranging from 0.3 km to 4.0 km. Twenty of these events occurred at distances below 2 km, and the mean migration distance was 1.5 km. We therefore assumed that tree frogs regularly move over distances less than 2 km and that they are likely to move between distances of 2-4 km. However, tree frogs are less likely to move between distances of 4-8 km and unlikely to exceed distances greater than 8 km. Hence, we structured our analyses into these four distance classes. We first examined correlations among the 32 variables plus geographic distance by calculating Spearman rank correlation coefficients (r_s) per distance classes in SPSS 15.0.0 (SPSS 2006). When $r_s \geq \pm 0.700$, we retained only one variable, and, in doing so, generated a reduced set of less related landscape variables for subsequent analyses. We retained geographic distance in all analyses, because we wanted to investigate whether landscape elements had more impact on tree frog gene flow than geographic distance alone.

To analyse the influence of the reduced set of landscape variables and geographic distance on F_{ST}-*values* per distance class, we computed multiple regression models (MRMs) with permutation testing using F_{ST}-*value*s as dependent and the reduced set of landscape variables and geographic distance as independent variables in PERMUTE! 3.4 alpha 9 (Legendre et al. 1994). This procedure has been

recommended for landscape genetic analyses by Balkenhol et al. (2009b). PERMUTE! provides different permutation-methods to assess the significance of regression coefficients and associated R^2-values on a dependent variable (Legendre et al. 1994). For two reasons, we selected the vector method with stepwise backward elimination (exclusion at $\alpha = 0.01$; 9999 permutations) that permutes the dependent variable at random. First, by analysing the pairwise genetic and landscape data in four distance classes, we generated incomplete triangular matrices that prohibited the execution of the matrix method in PERMUTE! The latter method would permute the matrix of the dependent variable as in a partial Mantel test. Second, our landscape variables were not actual distances, such as the pairwise F_{ST}-*value*s, as they consisted of relative proportions and densities.

We inspected the outcome of the MRMs in two separate ways. First, we checked the significant model variables for correlations of $r_s \geq \pm 0.600$ and sign consistency of their correlation coefficients with the F_{ST}-*value*s. Since both cases occurred in the two distance classes exceeding 4 km, corresponding models were re-computed by retaining only one of the correlated variables. Second, we repeated the permutation analyses per distance class by building MRMs of F_{ST}-*value*s against the complete set of 32 landscape variables and geographic distance. The significant variables in these MRMs were again examined for correlations of $r_s \geq \pm 0.600$ and the sign consistency of their correlation coefficients with the F_{ST}-*value*s. Each model was re-computed by retaining only one of the significantly correlated variables. This procedure was again only necessary in the models for distance classes exceeding 4 km. Note that in all cases, the variable with the higher correlation with F_{ST}-*value*s was retained in the MRMs.

Results

The eleven microsatellite loci exhibited no significant linkage disequilibrium, and only two breeding sites expressed significant deviation from Hardy-Weinberg equilibrium. The pairwise F_{ST}-*value*s among breeding sites ranged from -0.091 to 0.374, and the majority (72.4%) were significantly different from zero ($p < 0.05$). The geographic distances between breeding sites ranged from 0.28 km to 20.98 km. The numbers of pairwise data sets analysed in the four distance classes were $n = 114$ (< 2 km), $n = 109$ (2-4 km), $n = 63$ (4-8 km) and $n = 255$ (> 8 km). When checking for correlations of $r_s \geq \pm 0.700$, only DLE5 (building density) was omitted at all four distance classes,

while all other variables where further analysed in distance-class specific arrangements comprising 18 to 20 variables (Table 1). Geographic distance correlated with all landscape variables with r_s-values < ±0.700 except LE15 (buildings; r_s = -0.745) in the distance class exceeding 8 km, and this landscape element was therefore removed. The proportions or densities of the landscape elements LE1-LE6, LE8, LE9, LE12, LE14, LE15, DLE1-5, DLE7-11, DLE13, DLE15 and DLE16 were not significant in the MRMs loaded with either the reduced set or the complete set of landscape variables (Table 1). The proportions or densities of the significant landscape variables were generally small and ranged from zero to 0.1544 for LE7, from zero to 0.2740 for LE10, from 0.0076 to 0.5698 for LE11, from zero to 0.1205 for LE13, from zero to 0.0012 for LE16, from 2.4284E-06 to 54.0499E-06 for DLE6 from zero to 20.4206E-06 for DLE12 and from zero to 7.1227E-06 for DLE14, depending on the distance class considered.

The MRMs computed with the reduced set of landscape variables were highly significant for all distance classes and showed R^2-values ranging from 0.209 to 0.644. At the distance class of less than 2 km, LE7 (rivers or lakes) was positively, and LE13 (dry meadows or pastures) and LE16 (stepping stones) negatively related to F_{ST}-*value*s (Table 1). At the distance class of 2-4 km, LE10 (wetlands), LE11 (amphibian ponds) and geographic distance were positively related to F_{ST}-*value*s. At the distance class of 4-8 km, LE10 (wetlands), DLE6 (forest density) and geographic distance were positively, and DLE12 (hedgerow density) and DLE14 (protected area density) negatively related to F_{ST}-*value*s. At the distance class of greater than 8 km, LE11 (amphibian ponds) and LE13 (dry meadows or pastures) were positively, and geographic distance negatively related with F_{ST}-*value*s (Table 1). Six landscape elements had to be additionally removed in the analyses of the reduced data set because of correlation r_s > ±0.600 of these variables retained in the MRMs and sign inconsistencies with F_{ST}-*value*s. These were LE4 (trees) and LU6 (forests) at the distance class of 4-8 km and LE1 (roads), LE4 (trees), LE10 (wetlands), LE12 (hedgerows) and DLE11 (amphibian pond density) at the distance class of greater than 8 km. Note that the sign of the correlation of geographic distance and LE13 (dry meadows or pastures) with F_{ST}-*value*s changed depending on the distance class considered. Geographic distance showed a positive correlation with F_{ST}-*value*s at distance classes less than 8 km, but a negative correlation at the distance class of greater than 8 km (Figure 2), whereas LE13 (dry meadows or pastures) showed a

negative correlation with F_{ST}-*value*s at the distance class of less than 2km and a positive correlation at the distance class of greater than 8km (Table 1).

Of the eight variables appearing in the MRMs, five were highly correlated with landscape elements that were excluded during correlation analyses. At the distance class of less than 2 km, LE7 (rivers or lakes) was correlated with LE8 (river and lake edges; r_s = 0.839), while LE16 (stepping stones) was correlated with DLE16 (stepping stone density; r_s = 0.996). At the distance class of 2-4 km, LE10 (wetlands) was correlated with DLE10 (wetland density; r_s = 0.911), while LE11 (amphibian ponds) was correlated with LU3 (gravel-pits; r_s = -0.763), LE12 (hedgerows; r_s = -0.743), DLE1 (road density; r_s = 0.713) and DLE11 (amphibian pond density; r_s = 0.792). At the distance class of 4-8 km, LE10 (wetlands) was again correlated with DLE10 (wetland density; r_s = 0.912), while DLE12 (hedgerow density) was correlated with LE12 (hedgerows; r_s = -0.851).

The MRMs computed with the complete set of all 32 landscape variables and geographic distance versus F_{ST}-*value*s gave identical results to those calculated with the reduced set at distance classes of 2-4 km and greater than 8 km, and very similar results at the distance classes of less than 2 km and 4-8 km. At the distance class of less than 2 km, only LU7 (rivers or lakes) was retained in the final MRM (R^2 = 0.0995, p = 0.0001) when using the complete data set, whereas at the distance class of 4-8 km, only LE10 (wetlands), DLE6 (forest density), DLE12 (hedgerow density) and geographic distance were retained (R^2 = 0.5723, p = 0.0001).

Discussion

Our study in the Swiss river Reuss valley showed that *H. arborea* is influenced by different landscape elements depending on the spatial scale studied. At distance classes less than 2 km, the only inhibitory landscape element determining gene flow between breeding sites was the proportion of rivers or lakes (i.e. river Reuss; Figure 1). At this distance class, a supportive effect of the presence of stepping stones (i.e. other *H. arborea* breeding sites) on gene flow was apparent (Table 1). The first result confirmed a long-standing presumption of conservation practitioners, namely that the river Reuss forms a barrier to tree frog movement (Christoph Flory, Ennetbaden, pers. comment; Harald Cigler, Affoltern am Albis, pers. comment). The second result emphasized the need of a dense habitat network for the European tree frog, which is particularly effective when breeding sites are spaced at distances of less than 2 km.

A surprising result, however, was that geographic distance was not an explanatory variable of genetic differentiation at this spatial scale (Table 1). We interpret this result as clear indication that European tree frogs are readily able to cover distances up to 2 km on a regular basis without being substantially hindered by any structural element. This interpretation is compatible with earlier studies on *H. arborea*, where average dispersal distances of 1.5 km were detected from both mark-recapture data in the Netherlands (Vos et al. 2000) and from first-generation migration genetic analysis across the Reuss valley (Angelone & Holderegger 2009). Furthermore, a review on amphibian dispersal also suggested an average movement distance of 2 km for anurans (Smith & Green 2005).

At distances of 2-4 km, gene flow was no longer affected by either the proportions of rivers or lakes or of stepping stones. Instead, the inhibitory elements for gene flow were geographic distance and the proportions of wetlands and amphibian ponds (Table 1). The proportion of amphibian ponds was originally highly correlated with the proportions of gravel-pits and hedgerows, and the density of roads and amphibian ponds. The correlation between amphibian pond proportion and amphibian pond density is self-explanatory, while the correlations between the proportion of gravel-pits and hedgerows might be mutual. Gravel-pits are known as alternative breeding sites for many amphibian species, and amphibian ponds, in turn, are frequently surrounded by hedgerows (Stumpel & Tester 1993; Vos et al. 2007). However, the relationship with road density was less obvious. It is generally challenging to clearly disentangle the relative effects of highly correlated landscape elements (Balkenhol et al. 2009b). When re-computing the analysis of the distance classes of 2-4 km with a slightly modified reduced set of variables (i.e. amphibian pond density was replaced with each of the above mentioned correlated variables; Table 1), only road density, with geographic distance and wetland proportion, was retained as an explanatory variable (analysis not shown). Hence, the effects of road density can not be disentangled from those of the proportion of amphibian ponds in our study landscape. We were nevertheless able to detect the most influential inhibitory landscape variables on tree frog movement at distances of 2-4 km in the Reuss valley, which were the proportion of wetlands, amphibian ponds or road density, and geographic distance. However, no supporting landscape element was detected at this distance class.

The inhibitive nature of wetlands and amphibian ponds on tree frog gene flow at distances of 2-4 km is counter-intuitive at first glance. However, on closer examination, the reasons for the inhibiting nature of these two elements became evident. Tree frogs are socially attracted by chorus calls and therefore tend to disperse to already occupied ponds, thereby ignoring intermediate ponds (Vos et al. 2000). Additionally, the landscape variable wetlands mainly consisted of fens and peat bogs, which are qualitatively poor habitats for tree frogs because tree frogs require sunny standing water bodies containing sparse water plants and shallow areas to ensure warm water temperatures (Stumpel & Tester 1993). These water bodies should ideally be surrounded by meadows or pastures structured by woodlots, hedgerows or forest edges, offering summer and hibernation habitats for tree frogs (Tester & Flory 1995). The European tree frog is thus considered a demanding species concerning its habitat selection. Protected amphibian areas for species other than the tree frog might therefore be unsuitable or even form competitive areas because of high abundances of other amphibian species (Stumpel & Tester 1993; Pellet et al. 2004b). Hence, wetlands and amphibian ponds formed potentially unattractive areas negatively influencing tree frog movement in the Reuss valley, which was additionally hindered by road density. It is noteworthy that road density was exclusively correlated with the proportion of amphibian ponds at distances of 2-4 km. In a concentric analysis focussing on tree frog presence, a clear impact of road and traffic density was found in Western Switzerland (Pellet et al. 2004a), and it was therefore surprising that road density and other landscape elements related to urbanization, such as buildings, did not show a clearly inhibiting impact on gene flow across distance classes (Table 1). However, a recent meta-analysis on road mortality of eleven amphibian species found unexpectedly low road-kill records for *H. arborea* in Europe (Elzanowski et al. 2009), and a radio telemetry study observed that tree frogs can successful cross roads (Pellet et al. 2006). We interpret these results as suggesting that roads do not exert a particularly high inhibitive effect on recurrent gene flow in tree frogs.

At distances of 4-8 km, forests emerged as an additional explanatory element inhibiting tree frog movement, along with the proportion of wetlands and geographic distance, whereas the densities of hedgerows and protected areas appeared as supportive elements for gene flow (Table 1). Many amphibian species tend to avoid dry or arable landscapes and prefer structured habitat, such as hedgerows, which

offer a broader spectrum of food resources (Vos et al. 2007). It is plausible that movement and dispersal will be favoured in a landscape that provides a high density of suitable habitat (Mazerolle & Desrochers 2005; Stamps et al. 2005). Several studies on tree frog movement, however, reported that they rarely move more than 4 km in fragmented landscapes (Vos et al. 2000; Andersen et al. 2004; Arens et al 2006; Angelone & Holderegger 2009). The presence of geographic distance as an explanatory variable inhibiting tree frog gene flow at distances greater than 4 km might indicate an approximation of the dispersal limit of the species at the spatial scale of 4-8 km. This could also give an explanation for the negative influence of extended forests on tree frog movement.

When distances exceeded 8 km, the proportions of amphibian ponds, dry meadows or pastures and geographic distance emerged as explanatory landscape elements of gene flow (Table 1). The effects of the latter two variables were opposite to those of the same variables at smaller distance classes (Table 1). Furthermore, the slope of the relationship of geographic distance with pairwise F_{ST}-*value*s changed from being clearly positive to slightly negative at about 8 km (Figure 2). The same isolation by distance pattern has been found in an earlier study on *H. arborea* in The Netherlands at an almost identical spatial scale (1.2-24.1 km; Fig. 2 in Arens et al. 2006). This pattern may well reflect the differing roles of gene flow and genetic drift over different spatial scales, namely that gene flow was more effective among populations separated by shorter distances, whereas genetic drift was more influential in populations separated by larger distances (case IV in Fig. 1 in Hutchison & Templeton 1999). The observed pattern between F_{ST}-*value*s and geographic distance is thus a clear indication that distances exceeding 8 km represent a spatial dimension that is rarely surpassed by tree frogs, although a maximal dispersal distance of 12.6 km has been recorded for this species (Smith & Green 2005). Such long-distance dispersal events, however, are considered to be exceptional for tree frogs as well as other amphibians (Smith & Green 2005; Arens et al. 2006). Therefore, tree frog breeding sites are only loosely connected by occasional gene flow at a spatial scale exceeding 8 km. In consequence, this might imply that, at larger distances, explanatory landscape variables lose their predictive value as the rarity of long-distance dispersal events limits the statistical power to assess real landscape effects (Van Dyck & Baguette 2005; Lowe 2009). It is even doubtful

whether landscape effects on dispersal and movement at large spatial scales actually exist.

Our analyses of genetic differentiation among *H. arborea* breeding sites in the Swiss Reuss valley gave consistent results when applying both the complete or reduced sets of landscape variables. As a synthesis, we propose that tree frogs frequently cover distances of up to 2 km and are thereby only hindered by substantial natural barriers such as the river Reuss. At distances between 2 and 4 km, tree frogs start to perceive movement costs. Consequently, their movement becomes more selective and dependent on high quality habitat. At distances between 4 and 8 km, tree frogs reach their dispersal limit and avoid crossing landscapes of high resistance such as forests. Hence, a combination of measures enhancing both the quality of tree frog breeding sites and the connectivity among them would be a successful strategy for tree frog conservation. We recommend that conservation practitioners continue pursuing this strategy under the consideration that a functional habitat network for European tree frogs in fragmented landscapes must have maximum mesh widths of 2 km.

In conclusion, our study showed that the spatial scale at which landscape genetic analyses are conducted is pivotal. Gene flow is largely determined by the dispersal abilities and movement frequencies of the study organisms, and the landscape elements influencing gene flow are likely to change depending on the particular landscape studied. The composition of each landscape is complex and varies in space and time (Balkenhol et al. 2009a). In landscape genetic analyses, the interactions between gene flow and landscape shift between scales and should not be analysed in a single overall analysis. We propose that study should be conducted at spatial dimensions in which migration and dispersal are likely to occur, because pooling data over scales that differ in movement probabilities might cause misleading results. Hence, researchers should increasingly draw their attention to the spatial scales that their study organisms are likely to experience during dispersal and movement and should analyse gene flow data in relation to landscape elements at correspondingly accurate scales. Since our study clearly points to the importance of scale in landscape genetics, we encourage researchers to test for novel ideas and statistical approaches.

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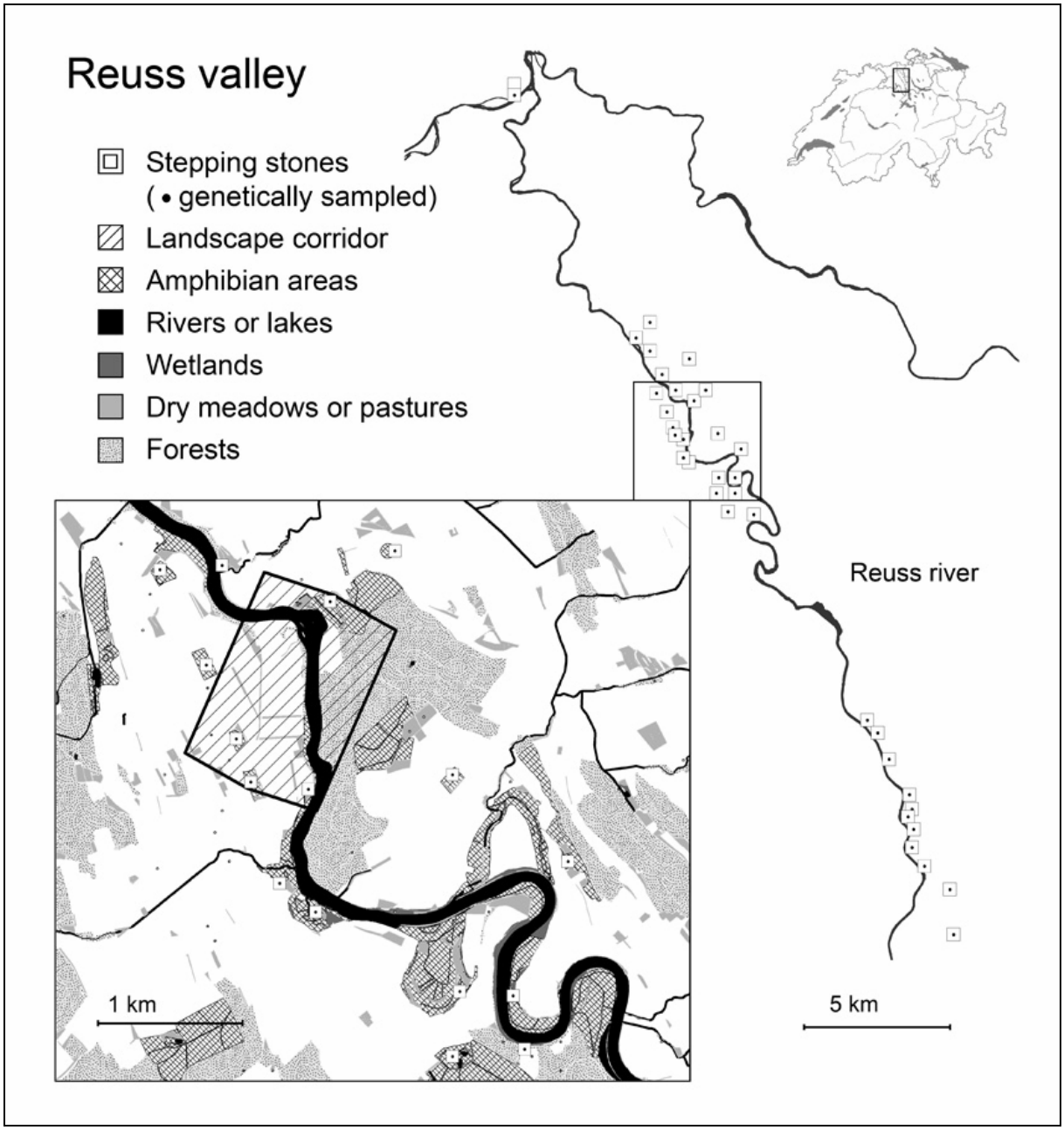

Figure 1. Localisation of the 36 studied breeding sites (i.e. stepping stones) of *Hyla arborea* in the Reuss valley in Switzerland (small inlet), of which 34 were genetically sampled. In the enlarged section, a detailed example of the location of a corridor area and significant landscape elements from landscape genetic analysis is given.

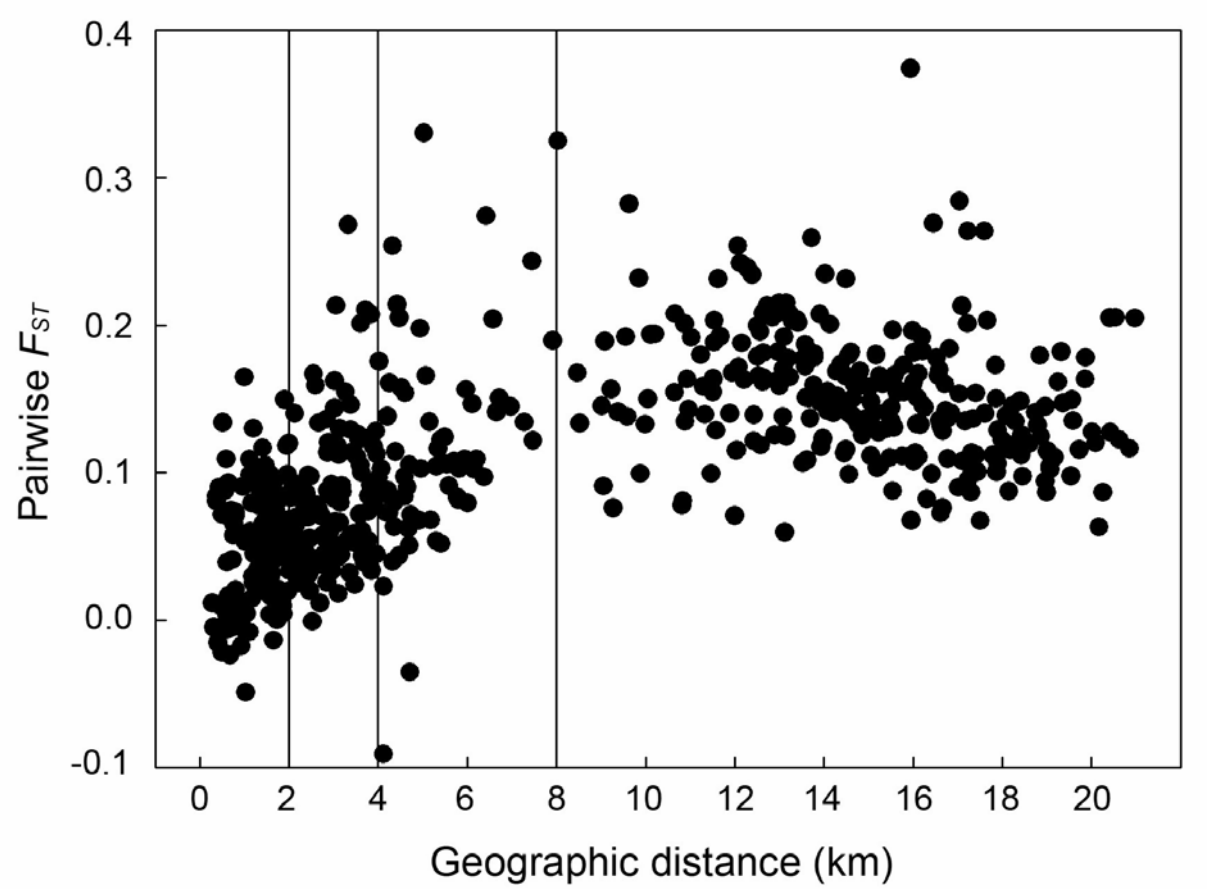

Figure 2. Pairwise F_{ST}-*value*s against pairwise geographic distances between 34 genetically analysed *Hyla arborea* breeding sites in the river Reuss valley in Switzerland. Vertical lines designate the four distance classes studied.

Table 1. Regression models of pairwise F_{ST}-*value*s of *Hyla arborea* breeding sites against proportions and densities of 16 landscape variables and geographic distance using backward elimination procedures (9999 permutations; exclusion at α = 0.01). The landscape variables were assembled within corridors of 1 km width between breeding sites in the Swiss Reuss river valley (Fig. 1). For each distance class model, the overall R^2-value and *p*-value are given on top of the table, and the standardised regression coefficients *b* and the corresponding *p*-values are given in bold for variables kept in the final models. For explanations on landscapes variables see text and Appendix 1.

		Model <2 km R^2 **= 0.2094** p **< 0.0001**		**Model 2-4 km** R^2 **= 0.4280** p **< 0.0001**		**Model 4-8 km** R^2 **= 0.6440** p **< 0.0001**		**Model >8 km** R^2 **= 0.2871** p **< 0.0001**	
Variables	**Code**	***b***	***p***	***b***	***p***	***b***	***p***	***b***	***p***
Geographic distance	DIST			**0.256**	**0.001**	**0.363**	**0.000**	**-0.301**	**0.000**
Proportion:									
Roads	LE1	na	na					na*	na*
Trails	LE2			na	na				
Gravel-pits	LE3	na	na	na	na			na	na
Trees	LE4							na*	na*
Forest edges	LE5					na*	na*		
Forests	LE6					na	na		
Rivers and lakes	LE7	**0.397**	**0.000**	na	na	na	na	na	na
River edges	LE8	na	na			na	na	na	na
Ponds	LE9	na	na						
Wetlands	LE10	na	na	**0.484**	**0.000**	**0.781**	**0.000**	na*	na*
Amphibian ponds	LE11			**0.319**	**0.000**			**0.337**	**0.000**
Hedgerows	LE12	na	na	na	na	na	na	na*	na*
Dry meadows or pastures	LE13	**-0.249**	**0.002**					**0.319**	**0.000**
Protected areas	LE14	na	na						
Buildings	LE15			na	na			na	na
Stepping stones	LE16	**-0.261**	**0.002**	na	na			na	na
Density:									
Roads	DLE1			na	na	na	na		
Trails	DLE2	na	na					na	na
Gravel-pits	DLE3								
Trees	DLE4	na	na	na	na	na	na		
Forest edges	DLE5					na	na	na	na
Forests	DLE6					**0.518**	**0.001**		
Rivers and lakes	DLE7	na	na	na	na			na	na
River edges	DLE8								
Ponds	DLE9			na	na	na	na	na	na
Wetlands	DLE10			na	na	na	na	na	na
Amphibian ponds	DLE11	na	na	na	na	na	na	na*	na*
Hedgerows	DLE12			na	na	**-0.564**	**0.000**		
Dry meadows or pastures	DLE13			na	na				
Protected areas	DLE14					**-0.369**	**0.001**		
Buildings	DLE15	na	na	na	na	na	na	na	na
Stepping stones	DLE16	na	na			na	na		

na: Landscape variables that were a priori excluded from model analyses because of significant correlations with other variables (rs > ±0.700; reduced data set)

na*: Landscape variables that were additionally excluded from model analyses because of significant correlations with other variables (rs > ±0.600) or sign inconsistencies of correlation coefficients in model evaluation

Appendix 1. List of the 33 land cover data assembled within corridors of 1 km width between *Hyla arborea* breeding sites in the Swiss Reuss valley and their pooling into 16 landscape element classes (bold). For each class, the coding for the proportion and density of patches within corridors is given.

Landscape elements	Proportion Code	Density Code
Roads	LE1	DLE1
Minor roads (two-sided 2.5m buffer)		
Motorways and main roads (two-sided 3 m buffer)		
Railways (two-sided 4m buffer)		
Trails	LE2	DLE2
Minor road (two-sided 2m buffer)		
Trails and agricultural land roads (two-sided 1m buffer)		
Gravel-pits	LE3	DLE3
Gravel- and loam-pits		
Rocks		
Tank training areas (two-sided 10m buffer)		
Trees	LE4	DLE4
Fruit trees and commercial tree plantings (2m buffer radius on single trees)		
Groups of trees (single tree resolution)		
Forest edges	LE5	DLE5
Fallow land		
Forest edges (5m buffer adjacent to forest border)		
Shrubs		
Forests	LE6	DLE6
Closed forests		
Riparian forests, wet forests and sparse forests		
Rivers or lakes	LE7	DLE7
Lake (standing water)		
Rivers (two-sided 10m buffer)		
Creeks (two-sided 1m buffer)		
River edges	LE8	DLE8
River edges (5m buffer adjacent to running river water)		
Creek edges (2m buffer adjacent to running creek water)		
Ponds	LE9	DLE9
All ponds (not necessarily protected for amphibians)		
Wetlands	LE10	DLE10
Fens		
Peat bogs		
Wet areas		
Protected wetlands		
Amphibian ponds	LE11	DLE11
Protected amphibian ponds (not necessarily of *H. arborea*)		
Hedgerows	LE12	DLE12
Hedgerows (two-sided 2m buffer) and tree rows (two-sided 2m buffer)		
Dry meadows or pastures	LE13	DLE13
Dry meadows		
Dry pastures		
Extensively used meadows		
Protected areas	LE14	DLE14
All remaining compensation and nature protection areas		
Buildings	LE15	DLE15
Buidlings (single building resolution)		
Stepping stones	LE16	DLE16
All known *H. arborea* breeding sites		

CHAPTER 3 – Article submitted to Biological Conservation

Are differences in fitness traits related with genetic clusters? An empirical test on the European tree frog

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Abstract. Microsatellites are the preferred markers to estimate the level of genetic diversity and subdivision in natural populations of endangered species. Many studies thereby seek to identify genetically defined management units for conservation by applying Bayesian genetic clustering methods. It is not clear, however, whether genetic clusters inferred from neutral molecular markers reflect differences in fitness or adaptation. In this study, I conducted a common garden experiment on the endangered European tree frog (*Hyla arborea*) to clarify whether fitness-related traits of larval development differed between three genetic groups defined by Bayesian clustering analyses. I reared larvae under semi-natural conditions and measured growth and developmental rates as well as survival at five larval stages from eclosion to completion of metamorphosis. Nested general and generalized linear models showed significant cluster differences for two variables in terms of smaller growth rates and body sizes at early larval stages. These differences were probably not linked to adaptive divergence among clusters but rather to neutral genetic processes in the populations of one cluster, which were spatially isolated and subject to recent bottlenecks. Hence, a genetic load effect (inbreeding depression) may have acted on the populations of this particular cluster. I advise studies aiming to define management units for conservation to not only use genetic clustering methods but to complement their findings with experimental approaches on fitness-related traits.

Key words: Conservation, fitness traits, *Hyla arborea*, microsatellites, species management, STRUCTURE software

Introduction

Microsatellites have become the preferred markers for conservation biologists and ecological geneticists to estimate the genetic diversity of populations of endangered species (Selkoe & Toonen 2006). Genetic diversity is one of three fundamental levels of biodiversity and a decrease in genetic variation can lead to increased inbreeding and reduced fitness within populations (Allendorf & Luikart 2007). However, it is controversial whether patterns of neutral genetic variation reflect population fitness and/or adaptive genetic variation (Merilä & Cronkrak 2001; Reed & Frankham 2001, 2003). Individual components of fitness, such as survival or disease resistance, are difficult to measure in natural populations and it is unclear whether such fitness components are correlated with neutral estimates of genetic variation (David 1998; Balloux et al. 2004). Although many studies have addressed the existence of multilocus heterozygosity-fitness correlations, the empirical results are heterogeneous (Coltman & Slate 2003; Grueber et al. 2008). There are two main explanations for heterozygosity-fitness correlations at neutral markers discussed in the literature. The local effect hypothesis states that heterozygosity-fitness correlations result from physical linkage of fitness relevant genes and marker loci on chromosomes (Hansson & Westerberg 2002), while the general effect hypothesis assumes that heterozygosity-fitness correlations result from the effects of inbreeding due to genome-wide homozygosity (Slate & Pemberton 2002).

In parallel, a plethora of studies on the genetic subdivision or differentiation of natural populations has accumulated in the literature. In many recent studies, Bayesian clustering methods – in particular the software STRUCTURE – have been used to detect groups of populations based on neutral multilocus genotype data (Pritchard et al. 2000; Kaeuffer et al. 2007). It is thereby often anticipated that the inferred genetic groups or clusters refer to appropriate management units in conservation management (Hampton et al. 2004; Schwartz & McKelvey 2009). However, the application of genetic clustering methods in conservation biology is controversial, as the detected clusters are based on neutral genetic variation and might not reflect differences in fitness or adaptive genetic variation (Manel et al. 2005; Kaeuffer et al. 2007). In other words, are the clusters inferred from neutral genetic markers effective management units? Empirical studies testing the existence of relevant differences in fitness or adaptive genetic variation among such clusters are warranted because genetic clusters, as defined in STRUCTURE analyses, are

increasingly used in conservation management of endangered species. The fact that the differentiation of populations measured at quantitative traits is often larger than the genetic differentiation of populations at neutral loci (Reed & Frankham 2001; McKay & Latta 2002) suggests that differences in fitness among clusters inferred from STRUCTURE analysis could indeed occur.

Amphibians possess characteristics that are useful for investigating neutral and adaptive genetic variation or fitness, and have therefore been the subject of several empirical studies investigating both population clustering or heterozygosity-fitness correlations (Beebee 2005). The results showed that amphibians tend to exhibit high levels of population subdivision (Palo et al. 2004; Allentoft et al. 2009) and that significant heterozygosity-fitness correlations occurred in endangered species such as the European tree frog (Andersen et al. 2004) or the Italian agile frog (Pearman & Garner 2005) as well as in common species such as the common frog (Lesbarrères et al. 2005) or the wood frog (Weyrauch & Grubb 2006). Studies on heterozygosity-fitness correlations have usually investigated individuals from populations that either differed in census size (Rowe & Beebee 2003), degree of spatial isolation (Sagvik et al. 2005), or habitat conditions (e.g. salinity; Gomez-Mestre & Tejedo 2004). However, no study on anurans has yet examined whether inferred STRUCTURE clusters show significant differences in fitness-related life-history traits. Amphibians are suffering a worldwide decline due, amongst other factors, to human-induced habitat fragmentation (Becker et al. 2007) so it is a timely task to address this question.

I conducted a common garden experiment with larvae from European tree frog (*Hyla arborea* L.) families originating from three STRUCTURE clusters inferred in a previous study based on eleven neutral microsatellite loci (Angelone & Holderegger 2009). I tested the hypothesis that larvae from different genetic clusters exhibit relevant differences in fitness traits. It is generally assumed that raising amphibian larvae under controlled conditions reduces the background noise of environmental heterogeneity and maximises the expression of genetic effects on individual fitness (Laugen et al. 2002). Larval developmental and survival rates, and the timing of, and size at, metamorphosis are widely accepted fitness attributes in amphibians (Beebee 2005). The latter two live-history traits are especially considered to have long term implications on the fitness of the terrestrial stage of amphibians (Berven 1990; Altwegg & Reyer 2003). Therefore, I conducted my experiment from eclosion until

completion of metamorphosis and measured the size, growth and survival of larvae reared under favourable semi-natural conditions. In general, induced stress tends to result in marked differences in fitness, as only few loci are involved in the reaction (Armbruster & Reed 2005). In contrast, trait differences detected under less stressful, or even favourable, environmental conditions may indicate that a wider range of loci are usually involved in the reaction (Keller & Waller 2002; Armbruster & Reed 2005).

Methods

Study species and area

The European tree frog (*H. arborea* L.) is listed as an endangered species in Switzerland, where massive habitat destruction and fragmentation has lead to a drastic reduction of the species' distribution (Angelone & Holderegger 2009). In the Reuss river valley in Switzerland, tree frogs in 1991 had become extinct in half of the tree frog breeding sites found in the early 1980s (Tester & Flory 2004). Angelone and Holderegger (2009) analyzed 582 individuals from 34 of the currently 36 occupied sites in the Reuss valley with eleven microsatellite loci, identified six STRUCTURE clusters and found a positive relationship between heterozygosity and chorus size. In the present study, I used individuals from three of these six clusters in a common garden experiment (Fig. 1). These were the clusters 2 and 3 located on opposite sides of the river Reuss and the spatially isolated cluster 6 (Angelone & Holderegger 2009). I selected two large breeding sites per cluster: Aegerten and Boesimoos in cluster 2 (30 and 90 calling males), Hard and Kraehhuebel in cluster 3 (>100 and 70 calling males) and Gmeimatt and Lorzespitz in cluster 6 (50 and 35 calling males; Angelone & Holderegger 2009). The distances among these sites ranged from 0.6-3.9 km within clusters and from 2-20 km between clusters.

Experimental design and data collection

I collected five to six egg masses of *H. arborea* of similar stage at each site during three consecutive days in May 2008. *H. arborea* females usually lay their clutches in several discrete servings (each clutch with 20 to 50 eggs) that are most likely sired by a single father (Friedl & Klump 2005). To reduce the risk of sampling pseudo-replicates, I thus sampled egg masses from different close lying ponds per breeding site whenever possible. Egg masses were kept indoors at room temperature until eclosion.

I subsequently reared the larvae outdoors under semi-natural conditions in 70 plastic pools (88 x 58 x 29 cm, Faserplast, Switzerland) on a lawn at the University of Zürich. The pools comprised 80 l of tap water and were stocked with dried leaf litter, rabbit chow, pond water and a diverse community of zooplankton and algae following Van Buskirk (2002). The pools were installed three weeks before tadpole insertion to enable algae growth providing the tadpoles with food. When the tadpoles had absorbed their yolk bag and were free swimming (Gosner stage 25; Gosner 1960), I stocked the pools with tadpoles. Each of the 35 families were reared separately and for each pool, I randomly selected eight tadpoles per family which resulted in a density similar to that observed in natural ponds (Van Buskirk 2005). To prevent excessive algae growth, I added a small *Limnea* snail (2-3 cm) to each pool and afterwards removed *Limnea* spawn as it occured. This procedure was repeated, resulting in two experimental blocks each with 35 pools. Note that there were no differences in body size of tadpoles at hatching, neither for STRUCTURE clusters nor for populations nested within clusters ($F_{2,3}$ = 0.283, p = 0.756; $F_{3,28}$ = 1.538., p = 0.227, respectively).

I recorded the weight and age of the tadpoles at five stages: (1) at pool stocking, (2) on day 14 after stocking, (3) on day 28 after stocking, (4) on the day of emerging forelimbs (Gosner stage 42; Gosner 1960), and (5) on the day of completed tail resorption (Gosner stage 45: Gosner 1960). At stage (1), I recorded the mean weight of five tadpoles per family. At stages (2) and (3), I recorded the mean weight of five randomly selected tadpoles per pool. At stage (4), I daily checked the pools for metamorphs, which, in the case of occurrence, were removed, weighed and subsequently kept individually in transparent plastic boxes (192 x 129 x 95 mm, Superfos, Denmark), stocked with leaves and water. At stage (5), I daily checked these plastic boxes for froglets that had completed tail resorption, which where then weighed. I released all froglets at their original sampling site at the end of the experiment. Note that the dates of stages (1) to (3) were fixed while dates varied for stages (4) and (5) because they relied on individual development.

Data analysis

I calculated the following estimates of fitness related traits per family (i.e. mean values per pool): Weight (g) as a surrogate of body size at stages 1-5, time (d) for the development from stages 1-4 (hereafter time until metamorphosis), 4-5 (hereafter

time for metamorphosis) and 1-5 (hereafter time for total development), growth rates (g/d) for stages 1-2, 2-3, 3-4, 4-5, 1-3 and 3-5, developmental time rate (d^{-1}) until, and for, metamorphosis, total developmental time rate and percentage of survival until and during metamorphosis. In block 1, one family of cluster 6 and in block 2, four families from cluster 6 and a family from cluster 3 were accidentally lost during the experiment. Hence, I used a total of 512 tadpoles stemming from 34 families in block 1 and from 30 families in block 2 in the subsequent analyses.

I checked the variables for normal-distribution using Kolmogorov-Smirnov tests. All variables except time for metamorphosis and survival were normally distributed. Time for metamorphosis was normally distributed after log transformation, whereas survival was inconvertible. I calculated pairwise Pearson or Spearman correlation coefficients for all pairs of variables and only one variable was retained in the analyses in cases where correlation coefficients were greater than 0.700. For the normally distributed variables, a general linear model was fitted in a hierarchically nested analysis with STRUCTURE clusters, populations nested within STRUCTURE clusters, and families nested within populations within STRUCTURE clusters as random factors. The two replicates were introduced as a block effect. Exploratory analysis showed that all interactions were not significant. They were thus excluded from the models. In cases where the cluster factor was significant, I performed corresponding Tukey-Kramer post-hoc comparisons. For the variable survival, I fitted a generalized linear model that was structurally similar to the general linear models outlined above, with binomial error distribution and logit-link function. All analyses were performed using JMP 7.0 (SAS, Cary, United States).

To test whether the demographic decline of the 1980s caused detectable evidence of genetic bottlenecks, I used BOTTLENECK 1.2.02 (Piry et al. 1999). In recently bottlenecked populations, theory predicts that the observed heterozygosity will be higher than would be expected under mutation-drift equilibrium (Piry et al. 1999). I performed one-tailed Wilcoxon sign rank tests for heterozygosity excess per breeding site, using a two-phased model of mutation with 95% single-step mutations and 5% multi-step mutations, as recommended by Piry et al. (1999).

Results

Mean tadpole body size per family ranged from 0.013 to 0.025 g at stage 1, from 0.074 to 0.166 g at stage 2, from 0.390 to 0.902 g at stage 3, from 0.363 to 0.732 g

at stage 4 and from 0.225 to 0.475 g at stage 5 (Fig. 2). The mean time until metamorphosis ranged from 41.7 to 47.8 d, and the mean time for metamorphosis ranged from 3.0 to 5.6 d. Accordingly, the mean time for total development ranged from 44.9 to 52.6 d. Growth rates ranged from 0.004 to 0.010 g/d for stages 1-2, from 0.022 to 0.056 g/d for stages 2-3, from -0.026 to 0.009 g/d for stages 3-4, from -0.066 to -0.034 g/d for stages 4-5, from 0.018 to 0.042 g/d for stages 1-3, and from -0.032 to -0.003 g/d for stages 3-5. Developmental rates ranged from 0.021 to 0.024 d^{-1} until metamorphosis, from 0.180 to 0.333 d^{-1} for metamorphosis, and from 0.019 to 0.022 d^{-1} for total development. Total survival ranged from 62.5 to 100 %. Only two individuals died during metamorphosis. Mean tadpole body sizes and growth rates of all families increased at stages 1-3 and decreased at stages 4-5, except for two families originating from the site Boesimoos in cluster 3 (Figs. 1, 2). These two families increased in size and growth rate until stage 4.

There were strong correlations between several variables. Growth rate for stages 1-2 correlated with body size at stage 2 (r = 0.991) and with growth rate for stages 3-5 (r = -0.736). Growth rate for stages 1-3 correlated with body size at stage 3 (r = 0.984), with growth rate for stages 2-3 (r = 0.985) and with growth rate for stages 3-4 (r = -0.826). Developmental rate until metamorphosis was related to total developmental rate (r = 0.978), to time until metamorphosis (r = -0.999) and to total time (r = -0.975). Developmental rate for metamorphosis correlated with time for metamorphosis (r = -0.996), and body size at stage 5 correlated with body size at stage 4 (r = 0.949) and with growth rate for stages 4-5 (r = -0.736). Total survival exhibited no significant Spearman correlation with any other trait. Hence, I only kept the following six variables in the analyses: Growth rate for stages 1-2, growth rate for stages 1-3, developmental rate until metamorphosis, developmental rate for metamorphosis, body size at stage 5 and total survival.

The general linear models revealed considerable R^2-values ranging from 0.55 to 0.81. Families nested within populations within STRUCTURE clusters were significant for the traits growth rate for stages 1-2, growth rate for stages 1-3 and developmental rate until metamorphosis (Table 1). The factor cluster was significant for the traits growth rate for stages 1-2 and growth rate for stages 1-3, and marginally significant for body size at stage 5 (Table 1). The Tukey-Kramer comparisons revealed that cluster 6 was different from clusters 2 and 3 for growth rate for stages 1-2, whereas cluster 6 was different from cluster 2 but not from cluster 3 for growth rate for stages

1-3 and clusters 2 and 3 were not significantly different from each other (Table 1, Fig. 3). Traits in cluster 6 were generally smaller than in clusters 2 and 3 (Fig. 3). Only the generalized linear model for total survival was significant for the factor block, where survival in one experimental block was reduced in cluster 6 (Table 1, Fig. 3).

The Wilcoxon tests performed in BOTTLENECK revealed significant heterozygosity excess, and hence, evidence for bottlenecks, for the breeding sites Gmeimatt ($p \leq 0.$ 0.001) and Lorzespitz ($p = 0.042$), which were both situated in the isolated cluster 6. The breeding sites from clusters 2 and 3 showed no evidence of recent bottlenecks.

Discussion

I found significant differences in some fitness traits among *H. arborea* tadpoles originating from distinct STRUCTURE clusters. Initial growth rates of individuals from the spatially isolated cluster 6 were generally smaller than those from the better connected clusters 2 and 3 (Fig. 1). Furthermore, tadpoles from cluster 6 tended to exhibit an elongated development until metamorphosis of approximately one day and to have smaller body sizes across all stages measured, as reflected by the body size at stage 5 (i.e. completed metamorphosis), which was marginally different among clusters (Figs. 2, 3). In contrast, there were no significant differences for developmental rates, time elapsed until and for metamorphosis, and survival (Fig. 3). Survival was generally high, and slightly reduced survival was only evident in one experimental block of cluster 6 (Fig. 3). Given that differences in fitness traits are rather expressed in elevated stress situations than under favourable conditions (Armbruster & Reed 2005), it is noteworthy that I could actually detect fitness differences among STRUCTURE clusters.

Several effects can cause size and growth differences during the early life history stages of anurans. Environmental, maternal and genetic effects are most often discussed, and are usually difficult to disentangle (Pakkasmaa et al. 2003; Laugen et al. 2005; Lesbarrères et al. 2005). Significant size differences in fitness traits have been found in experiments on anuran larvae chosen from breeding sites that differ in the climatic or chemic environment (Laugen et al. 2002; Gomez-Mestre & Tejedo 2004), or in population sizes (Rowe & Beebee 2003). In the present case, the breeding sites where egg clutches had been sampled were situated at a rather small geographic scale in a flat river valley (Fig. 1). Furthermore, these breeding sites had been managed in a similar way and even had similar population sizes (Angelone &

Holderegger 2009). Besides, the tadpoles were reared under controlled environmental conditions, and since there were no block effects except for survival, local environmental effects during the experiment can be ruled out. A study on the Iberian spadefoot toad revealed variation in the metamorphic size of larvae reared in a common garden experiment that paralleled the variation found in adult body size in corresponding localities in the wild (Marangoni & Tejedo 2008). These localities were distributed at a similar geographical scale as in the present study, and the authors suggested that the observed geographic variation in metamorphic traits may have a genetic basis, but is mixed with maternal effects. However, the egg clutches I used in the experiment were deposited and fertilized in their natural environment, meaning that differences in environmental conditions could also have been transmitted by non-genetic maternal effects (Laugen et al. 2002).

Recent work on maternal effects revealed that they are much more complex and dynamic than previously thought. Maternal effects may alter with environmental or experimental condition and can be transposed to more than one generation (Räsänen & Kruuk 2007). In anurans, maternal effects are mainly expressed via egg size and are known to influence growth rates in larvae (Berven 1990). Laugen et al. (2005) suggested that the consequences of maternal effects vary significantly depending on the given experimental conditions and found that maternal effect x environment interactions are a significant source of variation in size and growth of *Rana temporaria* larvae. However, another study on common frogs from six different ponds revealed that egg size does affect larval growth and development, but that the size at metamorphosis is not affected by initial egg size because of a longer developmental time of larvae hatching from smaller eggs (Loman 2002). Loman (2002) also found significant pond effects indicating that common frog larvae originating from certain ponds metamorphosed earlier and at larger sizes than larvae from other ponds. These results are very similar to my results on *H. arborea* (Table 1, Fig. 3) except that mean hatch size did not differ between the breeding sites or clusters in the beginning of this experiment. Since I used larval growth rates that are known to be independent of egg size in amphibians (Kaplan 1989), I suggest that it is rather unlikely that the observed differences in growth rates were caused by maternal effects. The present differences in growth rates between tree frog clusters could rather have been caused by either differential adaptation to the environment or inbreeding effects. While I cannot rule out the existence of differences in adaptation

between clusters, there are several good reasons to assume that inbreeding effects caused the observed differences in growth rates.

The *H. arborea* breeding sites in the Reuss valley have experienced a drastic reduction in size and quantity, leading to the spatial isolation of cluster 6 (Fig. 1; Angelone & Holderegger 2009). As a matter of fact, only three of the eleven breeding sites of cluster 6 exhibited chorus sizes with more than ten calling males, while the smallest chorus sizes in both cluster 2 and 3 consisted of at least 20 calling males (Angelone & Holderegger 2009). Besides, the breeding sites in the Reuss valley exhibited a positive relationship between current male chorus size and the mean number of alleles and observed heterozygosity (Angelone & Holderegger 2009). It is widely accepted that small and isolated populations are more susceptible to inbreeding and genetic load effects (Allendorf & Luikart 2007). This was supported in that the tests preformed in BOTTLENECK reported evidence of genetic bottlenecks for the two breeding sites situated in cluster 6, while no bottlenecks were detected for the breeding sites of clusters 2 and 3 (Fig. 1). Genetic bottlenecks in European tree frog populations have been reported in The Netherlands (Arens et al. 2006) and Denmark, where the level of inbreeding (estimated as fixation indeces at 12 microsatellite loci) is positively related with larval mortality until hatching and metamorphosis, and fitness (Andersen et al. 2004). Hence, it is reasonable to assume that genetic load effects act in the studied breeding sites of cluster 6 and caused the differences in growth rates among clusters.

Several studies support the hypothesis that populations which are subject to inbreeding depression exhibit smaller sized offspring or adults (Coltman et al. 1998; Keller & Waller 2002; Fredrickson & Hedrick 2002; Wisely et al. 2008). Significant body size differences in anurans were found in the common frog, where the eggs from females from a large population crossed with males from a small and isolated population were significantly smaller and produced malformed tadpoles (Sagvik et al. 2005). In the natterjack toad, hatchlings originating from a small and isolated population were significantly smaller, grew slower and exhibited lower survival to metamorphosis when compared with hatchlings from a large, presumably outbred population (Rowe & Beebee 2003). A small body size in anuran larvae, especially at metamorphosis, is generally assumed to be associated with lower fitness in terms of post-metamorphic or adult growth and survival (Laugen et al. 2002; Lesbarrères et al. 2007, Marangoni & Tejedo 2008). Altwegg and Reyer (2003) state that even two

years old adults from metamorphs of larger body sizes show increased survival and faster growth when compared with adults from small metamorphs.

In conclusion, I found differences in fitness related traits among *H. arborea* larvae originating from different genetic clusters identified by STRUCTURE. These differences in life history traits are likely to be, at least to some extent, caused by genetic effects. However, this does not necessarily mean that the three clusters featured different local adaptation. In fact, these differences were rather triggered by recent demographic bottleneck events and the spatial isolation of cluster 6, potentially leading to increased inbreeding depression in the corresponding breeding sites. Hence, the results of this study do not justify the interpretation that the assignment of populations to genetic clusters was associated with fitness differences between clusters. Studies aiming to define management units for conservation based on genetic clustering of multilocus genotypes should therefore consider complementing their findings with experimental approaches on traits of fitness value in order to strengthen the evidence for substantial differences among clusters and therewith their relevance for conservation management. Understanding the processes behind potential cluster differences in fitness is important, as the latter do not necessarily point to differential adaptation but may also be caused by neutral processes (i.e. genetic drift), as in the present study. In other words, practical management decisions on wild populations are in need of information that goes beyond genetic clustering analyses.

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Table 1. General and generalized (in the case of total survival) linear models using a nested design (STRUCTURE cluster, populations within cluster, family within population within cluster (Fig. 1), and blocks (replicate) for six fitness traits in *Hyla arborea* larvae.

Trait	Factor	df	MS	F	p
Growth rate for stages 1-2	Cluster	2	5.96E-06	24.416	0.011
	Pop [Cluster]	3	2.37E-07	0.097	0.961
	Families [Pop [Cluster]]	28	2.52E-06	3.476	0.001
	Block	1	6.14E-08	0.085	0.773
Growth rate for stages 1-3	Cluster	2	3.23E-05	16.243	0.015
	Pop [Cluster]	3	1.83E-06	0.065	0.978
	Families [Pop [Cluster]]	28	2.87E-05	1.884	0.048
	Block	1	4.93E-06	0.324	0.574
Developmental rate until metamorphosis	Cluster	2	1.49E-06	3.449	0.165
	Pop [Cluster]	3	4.35E-07	0.697	0.562
	Families [Pop [Cluster]]	28	6.41E-07	2.663	0.005
	Block	1	8.20E-13	0.000	0.999
Developmental rate for metamorphosis	Cluster	2	5.10E-04	3.449	0.530
	Pop [Cluster]	3	6.50E-04	0.697	0.489
	Families [Pop [Cluster]]	28	8.00E-04	2.663	0.133
	Block	1	1.40E-04	0.000	0.614
Body size at stage 5	Cluster	2	2.68E-03	5.858	0.086
	Pop [Cluster]	3	4.50E-04	0.384	0.766
	Families [Pop [Cluster]]	28	1.18E-03	0.432	0.432
	Block	1	1.97E-03	0.192*	0.192
Total survival	Cluster	2	-	1.677*	0.432
	Pop [Cluster]	3	-	6.211*	0.102
	Families [Pop [Cluster]]	28	-	35.747*	0.149
	Block	1	-	4.408*	0.036

* χ^2 value

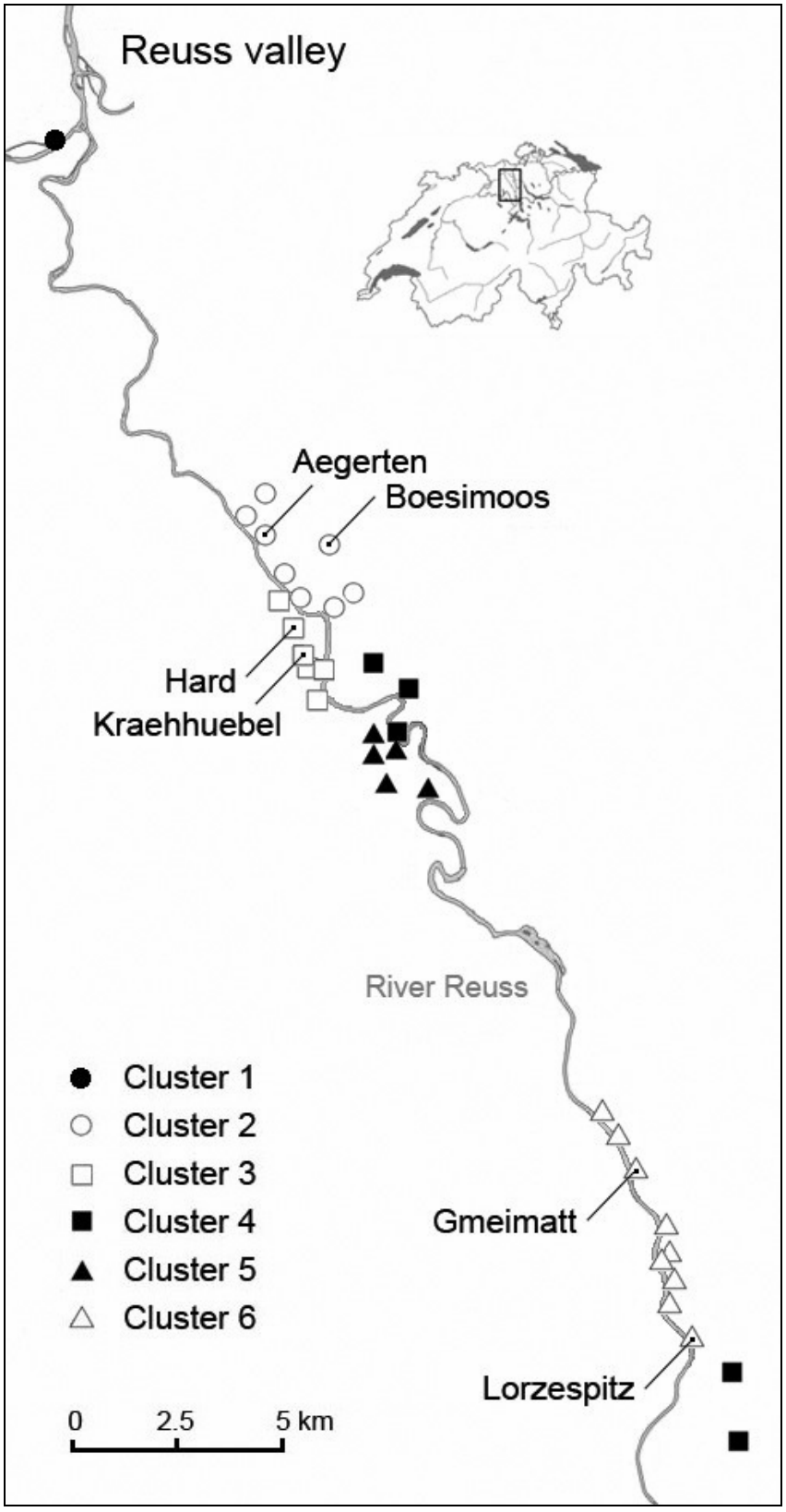

Figure 1. STRUCTURE clusters of genetically analysed *Hyla arborea* breeding sites in the Reuss valley in western Switzerland (Angelone and Holderegger 2009). Labelled sites are the source sites of egg clutches for the common garden experiment.

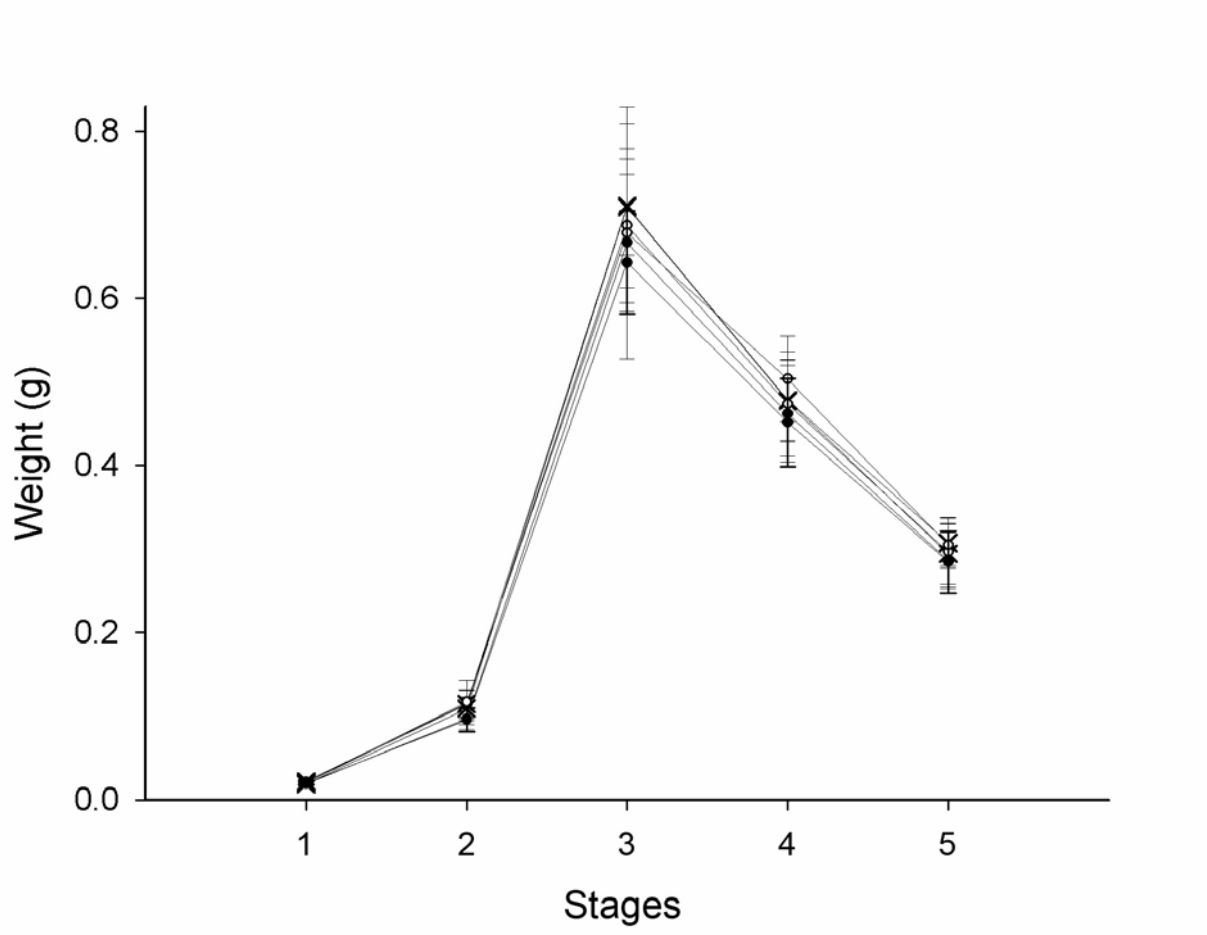

Figure 2. Change in body size of *Hyla arborea* larvae measured at five stages in a common garden experiment from the day of pool insertion to completed metamorphosis. Data are means ± SE across two blocks of three to six families from six populations sampled from three STRUCTURE clusters (Fig. 1). Open circles: Cluster 2; crosses: Cluster 3; filled circles: Cluster 6.

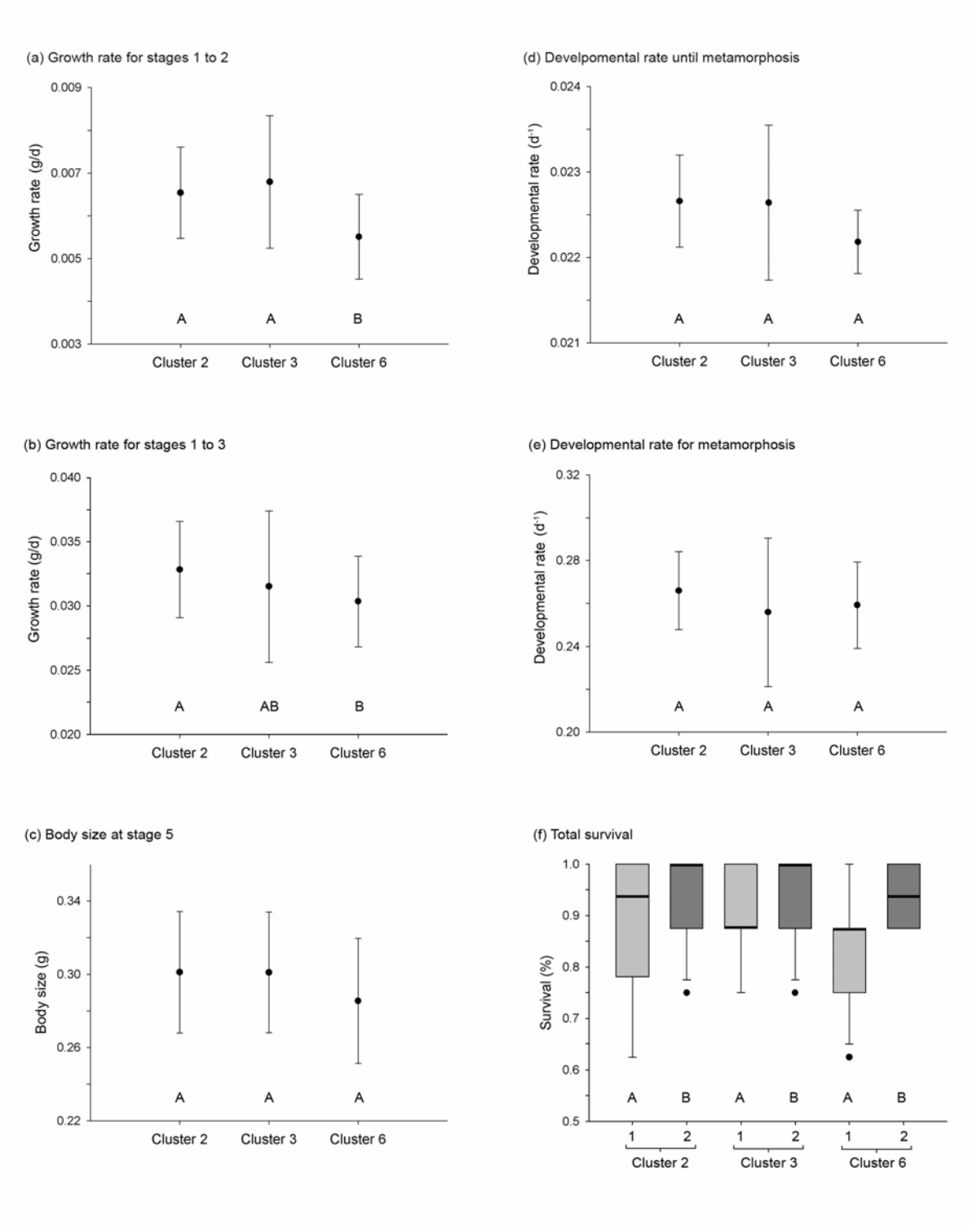

Figure 3. Variation in growth rates (a, b), body size (c), developmental rates (d, e), and survival (f) in a common garden experiment of *Hyla arborea* larvae from three STRUCTURE clusters. Data are means ± SE in (a) to (e) and Box-plots for two blocks (1 and 2) in (f). Different letters indicate significant differences among clusters according to Tukey-Kramer tests in (a) to (e) or among blocks in (f).

CHAPTER 4 – Article submitted to INSIDE

Laubfrosch und Vernetzungsprojekte: Eine Erfolgsgeschichte

Rainette verte et projets de réticulation: Une histoire à succèss

Sonia Angelone, Christoph Flory, Harald Cigler, Joggi Rieder-Schmid, Aline Wyss, Felix Kienast & Rolf Holderegger

Zusammenfassung. Schutzwürdige Arten und deren Lebensräume werden mit Mitteln der Gemeinden, Kantone und des Bundes im Rahmen von Vernetzungsprojekten gefördert. Diese sollen den Austausch von Individuen (und somit von Genen) zwischen den Restpopulationen in zerschnittenen Landschaften erhöhen. In einem interdisziplinären Projekt wurden unter Anwendung genetischer Methoden die für den Laubfrosch umgesetzten Vernetzungsmassnahmen im Reuss- und Thurtal auf ihren Erfolg hin überprüft. Die Resultate sind positiv und werden hier vorgestellt.

Résumé. Grâce aux moyens mis en oeuvre par les communes, les cantons et l'état fédéral, la protection des espèces et de leurs habitats prioritaires est favorisée par des projets de mise en réseau. Ce réseau devrait augmenter l'échange des individus (et par conséquence les flux de gènes) entre les populations restantes dans les paysages fragmentés. L'efficacité de telles mesures réalisées pour la rainette verte dans les vallées du Reuss et Thur a été vérifiée par des méthodes génétiques, au sein d'un projet interdisciplinaire. Le bilan est positif et les résultats principaux sont présentés dans ce rapport.

Landschaftszerschneidung und Vernetzung

Die Landschaften der Schweiz sind in den letzten Jahrzehnten vielerorts stark zerschnitten worden. Obwohl inzwischen Gegenmassnahmen getroffen wurden, stellen naturnahe Flächen nur noch isolierte Reste in einer intensiv genutzten Landschaft dar. Der Grad der Landschaftszerschneidung in der Schweiz ist besonders alarmierend, denn kaum ein anderes europäisches Land verfügt über eine solch hohe Dichte an Siedlungen und Verkehrsflächen. Die Landschaftszerschneidung konfrontiert Tierarten mit zahlreichen Problemen, insbesondere mit künstlichen Ausbreitungshindernissen wie Strassen, Bahnlinien, Siedlungen, Industriearealen und Landwirtschaftsflächen. Wenn solche Hindernisse nicht überwunden werden können, führt dies zur räumlichen und funktionalen Isolation der Bestände, was deren langfristige Überlebensfähigkeit beeinträchtigen kann. Um diesem schleichenden Prozess entgegenzuwirken, werden räumliche Verbindungen (Vernetzungen) als Ausbreitungshilfen zwischen ökologisch wertvollen Gebieten ausgeschieden oder neu gestaltet. Dies soll den Austausch von Individuen zwischen den Restpopulationen in zerschnittenen Landschaften erhöhen.

Normalerweise werden bei Vernetzungsprojekten zuerst die noch vorhandenen Habitatflächen gesichert und in Folge deren Qualität aufgewertet. Diese Flächen bilden sodann die Knoten eines Netzwerks, dessen Vernetzung mit zusätzlichen Massnahmen erhöht werden kann, indem dazwischen liegende Flächen des Netzwerks besser genutzt werden. Beispiele solcher Verbindungselemente sind Wildtierpassagen, Hecken, ökologische Ausgleichsflächen in der Landwirtschaft gemäss Ökoqualitätsverordnung (ÖQV) oder Trittsteinelemente wie Weiher. Lokal werden solche Massnahmen oft in Landschaftsentwicklungskonzepten (LEKs) dargestellt. So koordiniert etwa der Kanton Thurgau im Richtplan die Sicherstellung von Vernetzungskorridoren bei raumwirksamen Tätigkeiten. Diese Planungen beinhalten aber auch konkrete Vernetzungsmassnahmen für auserwählte Zielarten. Als Beispiel dazu stellen wir hier die spezifischen Artenschutzprogramme für den Laubfrosch in den Kantonen Aargau, Thurgau und Zürich vor.

Der Laubfrosch im Reuss- und Thurtal

Der Laubfrosch (Abbildung 1) mit seinem grossen Schau- und Sympathiewert ist eine Leitart der Auen. Seine Bestände sind in den 1980er Jahren im Schweizer Mittelland geradezu zusammengebrochen, was zum Aussterben des Laubfrosches in zehn

Kantonen führte. Um den Rückgang im unteren Reusstal aufzufangen, wurde ab 1992 das Projekt Laubfrosch in Zusammenarbeit von Pro Natura und dem Kanton Aargau umgesetzt. In den ersten Jahren wurden dabei 90% der noch vorhandenen Laichplätze des Laubfroschs vertraglich gesichert oder unter Schutz gestellt. Seit 1993 sind diese Laichplätze fortlaufend aufgewertet worden, um sie möglichst in einem Pionierzustand zu erhalten. Um die Ausbreitung des Laubfroschs im Lebensraumnetzwerk zu fördern, wurden seit 1993 zusätzliche Laichgewässer als Trittsteine geschaffen (Abbildung 2). In ähnlicher Weise wurden auch im oberen Reusstal im angrenzenden Kanton Zürich die Laubfroschvorkommen erfasst, deren Laichplätze gepflegt, aufgewertet und teilweise neu geschaffen. Zur Überwachung werden seit 1994 jährlich in beiden Kantonen die Laubfroschbestände entlang der Reuss überwacht, indem die Anzahl rufender Männchen an allen bekannten Beständen gezählt werden. Dank dieser Massnahmen zeigt sich im Reusstal die Situation für den Laubfrosch heute wieder erfreulicher: Der Rückgang wurde gestoppt und die Anzahl der Bestände ist seit Beginn der Massnahmen konstant geblieben. Insbesondere hat sich bis zum Jahr 2006 die Anzahl der erfassten Männchen im gesamten Reusstal mit rund 1100 rufenden Männchen mehr als verdoppelt.

Die grössten zusammenhängenden Laubfroschgebiete der Schweiz befinden sich in den Kantonen Schaffhausen, Zürich und Thurgau, insbesondere entlang der Thur, wo bereits in den 1980er Jahren viele Laichplätze unter Schutz gestellt wurden. Diese Schutzgebiete wurden seither gepflegt und die Massnahmen führten zu einer Stabilisierung der Laubfroschbestände. Seit 1999 sind einzelne Gebiete wie das Seebachtal oder die Frauenfelder Allmend aufgewertet worden und der Laubfrosch hat dabei die neu angelegten Trittsteingewässer spontan besiedelt. Im Gegensatz zum Reusstal existiert entlang der Thur aber kein Monitoring-Programm zur Überwachung der Grösse der Rufchöre. Zudem wurden konkrete Vernetzungs-massnahmen später ergriffen. Da die Laichplätze entlang der Thur aber seit längerer Zeit geschützt sind, widerspiegeln sie heute wohl am ehesten die ursprünglichen Verbreitungsverhältnisse des Laubfrosches in der Schweiz. Im Vergleich mit dem Thurtal lässt sich daher die Wirksamkeit von Vernetzungsmassnahmen im Reusstal testen.

Erfolgskontrollen

Für den praktischen Naturschutz ist es von grosser Bedeutung, dass der Erfolg umgesetzter Massnahmen überprüft wird. Erfolgs- oder Wirkungskontrollen für Vernetzungsprojekte verlaufen meist im Rahmen von Bestandeserfassungen, d.h. der Überprüfung der Zu- oder Abnahmen von Beständen. Bei solchen Erfolgskontrollen liegt der Nachteil darin, dass das eigentliche Ziel der Vernetzung, nämlich das Fördern des Austausches von Individuen und deren Erbgut zwischen Beständen, nicht evaluiert werden kann. Die Erfassung der funktionalen Vernetzung ist allerdings schwierig durchzuführen, weil umfangreiche Beobachtungen von wandernden Tieren, beispielsweise mittels Fang-Wiederfang-Studien, mit grossem Arbeitsaufwand und finanziellen Umtrieben verbunden sind. Im Falle des Laubfrosches haben sich die Bestände sowohl im Reuss- als auch im Thurtal stabilisiert oder vergrössert und neu angelegte Weiher wurden besiedelt. Aber heisst das auch, dass sich der Austausch von Laubfröschen zwischen den einzelnen Beständen verstärkt oder gar landschaftsweit etabliert hat? Mit der bisher verwendeten Monitoring-Methode lässt sich dies nicht mit Gewissheit bejahen.

Um diese Wissenslücke zu schliessen, führten wir in den letzten drei Jahren ein interdisziplinäres genetisches Kontrollprojekt durch. Dabei wurden folgende drei Fragestellungen untersucht, deren Hauptergebnisse anschliessend vorgestellt werden. Wie hoch ist der heutige Individuenaustausch (genetisch gesprochen, der aktuelle Genfluss) zwischen Laubfroschbeständen? Welche Landschaftsstrukturen beeinflussen den Austausch zwischen Beständen des Laubfrosches am stärksten? Weisen genetisch verschiedene Laubfroschbestände auch Unterschiede in ihrer Lebensfähigkeit auf?

Heutiger Individuenaustausch

In den ersten zwei Jahren wurde die genetische Zusammensetzung fast aller bekannten Bestände des Laubfrosches im Reusstal und eines Grossteils der Bestände im Thurtal untersucht (Abbildung 3). Insgesamt wurden rund 1200 Laubfrösche gefangen, um ihnen Mundabstriche für die genetische Untersuchung zu entnehmen und sie zu fotografieren, da sich die linienartige Zeichnung entlang ihrer Seiten als individuelles Erkennungsmerkmal eignet (Abbildung 1). Aus den Speichelproben wurden das Erbgut isoliert und anschliessend genetische Fingerabdrücke erstellt (bestehend aus 11 Mikrosatelliten). Nur in zwei Fällen haben

wir Laubfrösche gefangen, die jeweils identische genetische Fingerabdrücke aufwiesen. Die Seitenlinien zeigten, dass es sich dabei tatsächlich um Wiederfänge von zwei Einzeltieren handelte. Diese zwei Tiere bewegten sich jeweils in weniger als einem Monat über Distanzen von rund 1 km zu einem benachbarten Laichplatz.

Die Analyse der genetischen Fingerabdrücke zeigte sechs geographisch umrissene Gruppen im Reusstal (Abbildung 3). Wir führen dies darauf zurück, dass nach dem dramatischen Rückgang in den 1980er Jahren die in ihrer räumlichen Verteilung geschrumpften Laubfroschbestände eine Zeit lang voneinander isoliert waren. Später breiteten sich die Laubfrösche dann wieder aus, vorläufig jedoch ohne Durchmischung der Gruppen. Heute findet reger Individuenaustausch zwischen Beständen innerhalb der genetischen Gruppen im Umkreis von 2 km statt. Der Austausch zwischen Beständen unterschiedlicher Gruppen ist aber noch immer gering oder nicht vorhanden und begrenzt sich auf 4 km. Insbesondere fehlt wegen den räumlichen Verbreitungslücken von über 8 km jeglicher Austausch zwischen den Beständen im oberen und unteren Reusstal, sowie dem Bestand bei Brugg und dem restlichen Reusstal. Mit zunehmender Grösse der Laubfroschbestände ist zu hoffen, dass sich die genetischen Gruppen zwischen Mellingen und Bremgarten im unteren Reusstal in Zukunft stärker durchmischen werden.

Die Analyse der genetischen Fingerabdrücke im Thurtal zeigt hingegen ein ganz anderes Bild. Hier gehört die Mehrzahl der untersuchten Laubfroschvorkommen einer genetisch einheitlichen Gruppe an, die praktisch das ganze Thurtal umfasst. Nur zwei kleine Gruppen heben sich geographisch vom Hauptteil ab: Die Frauenfelder Allmend und der etwas isolierte Bestand bei Rickenbach (Abbildung 3). Diese hohe Ähnlichkeit der genetischen Fingerabdrücke erschwerte aber eine verlässliche Ermittlung des Individuenaustausches, denn dieser erstreckt sich anscheinend über grosse Distanzen von bis zu 16 km. Wir führen dieses diffuse Ausbreitungsbild darauf zurück, dass im Thurtal viele Laubfroschbestände dank der frühen Schutzmassnahmen erhalten geblieben sind und so auch ihre genetische Vielfalt bewahren konnten. Das Bild, das wir heute sehen, stellt aber nicht unbedingt die heutigen Ausbreitungsverhältnisse dar, sondern veranschaulicht vielmehr den beträchtlichen historischen Individuenaustausch zwischen den Beständen im Thurtal.

Relevante Landschaftsstrukturen

Der Individuenaustausch zwischen den einzelnen Laubfroschbeständen wird einerseits durch die vorhandene Landschaft und andererseits durch die Distanz zwischen den einzelnen Beständen beeinflusst. Der Umfang dieses Austausches wurde anhand einer landschaftsgenetischen Analyse im Reusstal abgeschätzt. Dabei wurden 33 Landschaftselemente innerhalb von 1 km breiten Korridoren, die paarweise zwischen allen Beständen gelegt wurden, räumlich explizit erfasst. Die aus der Analyse der genetischen Fingerabdrücke ermittelten Wanderdistanzen der Laubfrösche wurden in vier Klassen eingeteilt: Häufiger (0-2 km), wahrscheinlicher (2-4 km), seltener (4-8 km) und unwahrscheinlicher Austausch (über 8 km). In jeder Distanzklasse wurde die genetische Differenzierung zwischen den Beständen (ein Mass für Genfluss) mit den dazwischen liegenden Landschaftselementen und der geographischen Distanz einem multivariaten Analyseverfahren unterzogen. Diese Verfahren zeigten, welche Landschaftselemente hemmend oder fördernd auf den Gen- und somit Individuenaustausch des Laubfrosches einwirken.

Die Analysen zeigten im Bereich bis zu 2 km, dass Fliessgewässer einen hemmenden und Trittsteinelemente (andere Laubfroschgewässer) und Trockenwiesen hingegen einen fördernden Einfluss auf den Laubfroschaustausch ausübten. Im Bereich von 2-4 km hemmten die geographische Distanz, die Flächenanteile ungeeigneter Feuchtgebiete und Amphibiengewässer, sowie die Strassendichte den Austausch beim Laubfrosch. Im Bereich von 4-8 km erschienen die Distanz, sowie die Flächenanteile von Feuchtgebieten und die Wälderdichte als hemmende Elemente, während sich Hecken und Naturschutzflächen als fördernde Elemente erwiesen. Im Bereich über 8 km waren die Ergebnisse nicht aussagekräftig.

Unterschiedliche Lebensfähigkeit

Kleine und/oder räumlich isolierte Bestände sind anfälliger für den Verlust genetischer Vielfalt (beispielsweise durch Inzucht), was zu einer geringeren Lebensfähigkeit der Individuen führen kann. Bei Amphibien wird die Lebensfähigkeit mit Hilfe von Überlebens- und Wachstumsraten von Kaulquappen im Zeitraum vom Schlüpfen aus dem Laich bis zur Umwandlung zum terrestrischen Frosch sichtbar gemacht. Unter einheitlichen Aufzuchtsbedingungen werden dabei angeborene (genetische) Unterschiede zwischen verschiedenen Beständen messbar. Da die Bestände der räumlich isolierten Gruppe im oberen Reusstal eine deutlich reduzierte

genetische Vielfalt aufweisen (Gruppe 6; Abbildung 3), könnten diese Laubfrösche also über eine tiefere Lebensfähigkeit verfügen, als solche aus dem genetisch vielfältigeren Kerngebiet im unteren Reusstal (Gruppen 2-5; Abbildung 3). Wir haben deshalb die Lebensfähigkeit von Kaulquappen aus sechs Laichplätzen im oberen und unteren Reusstal getestet. An jedem Laichplatz wurden 5-6 Laichballen von verschiedenen Müttern gesammelt und jeweils acht der daraus geschlüpften Kaulquappen in 70 Einzelbecken im Freiland eingesetzt. Während der Aufzucht wurden die Kaulquappen regelmässig gewogen, um durchschnittliche Körpergewichte, Wachstums- und Entwicklungsraten, sowie Überlebenswahrscheinlichkeiten zu berechnen. Am Ende des Versuches wurden die Kaulquappen auf den Befall von Chytridiomykose getestet, und da dieser Test negativ ausfiel, wurden alle Tiere wieder an ihrem Herkunftsort freigelassen.

Wir fanden statistisch signifikante Unterschiede in den anfänglichen Wachstumsraten zwischen den genetischen Gruppen. Die Kaulquappen aus der isolierten Gruppe 6 wiesen durchschnittlich um 17% geringere Wachstumsraten über die ersten 14 Tage und 6% geringere Raten über die ersten 28 Tage auf als Kaulquappen von den weit besser vernetzten und genetisch vielfältigeren Laichplätzen aus dem unteren Reusstal. Kaulquappen aus Gruppe 6 neigten ausserdem zu längeren Entwicklungszeiten bis zur Metamorphose und zu geringeren Körpergewichten vor allem am Ende der Metamorphose. Die Überlebenswahrscheinlichkeit von durchschnittlich 90% fiel allerdings über alle Laichplätze hoch aus.

Erfolgsgeschichte der Praxis

Da die Laubfroschbestände im Reusstal noch immer eine klare räumlich-genetische Strukturierung aufweisen, ist eine landschaftsweite Vernetzung noch nicht erreicht worden. Trotzdem waren die verschiedenen getroffenen Vernetzungsmassnahmen für den Laubfrosch sehr erfolgreich, denn im Reusstal herrscht innerhalb der genetischen Gruppen reger Individuenaustausch, insbesondere im Umkreis von 2 km (Abbildung 3). Im Thurtal hingegen waren vorrangig die frühen Schutzmassnahmen erfolgreich, dank denen ein Grossteil der heutigen Bestände eine vermutlich noch immer verbundene Einheit darstellt. Da die Bestände im Thurtal mit durchschnittlich 2.8 km weiter auseinander liegen als jene im Reusstal, sollte hier allerdings der Austausch von Laubfroschindividuen durch gezielte Vernetzungsmassnahmen längerfristig sichergestellt werden. Die Naturschutzstrategie, durch strukturelle

Vernetzung eine funktionale Vernetzung herbeizuführen, war in beiden Regionen erfolgreich: Passende, neu geschaffene Trittsteingewässer werden von den Laubfröschen gut angenommen, rasch besiedelt und durch Individuenaustausch ins Lebensraumnetzwerk eingebunden.

Die detaillierte Landschaftsanalyse im Reusstal zeigte, dass Distanzen von unter 2 km zwischen Laichgewässern von Laubfröschen regelmässig überwunden werden, ausser sie treffen auf grosse, natürliche Barrieren wie die Reuss. Elemente des Siedlungsraumes scheinen dabei eine untergeordnete Rolle zu spielen. Entscheidend für die Vernetzung beim Laubfrosch ist das Angebot von qualitativ hochwertigen Laichgewässern im Umkreis von 2-4 km. Bei längeren Wanderdistanzen reagieren die Laubfrösche jedoch empfindlicher auf Strassen und Wälder, sowie weitere Landschaftselemente, auf die hier nicht weiter eingegangen wird. Da Hecken und Naturschutzgebiete einen positiven Einfluss auf den Genfluss zwischen Laubfroschgewässern im Abstand von 4-8 km ausübten, bilden Struktur gebende Landschaftselemente Ausbreitungshilfen für Laubfrösche über längere Distanzen. Räumliche Distanzen von über 8 km werden von den Laubfröschen nicht mehr überwunden.

Es ist bemerkenswert, dass mit einem einfachen Aufzuchtsexperiment relevante Merkmalsunterschiede wie kleinere Wachstumsraten zwischen genetischen Gruppen von Laubfroschbeständen gefunden wurden. Bei Fröschen hat eine kleinere Körpergrösse bei der Metamorphose negative Einflüsse auf die Lebensfähigkeit der Jung- und Erwachsenentiere. Unser Resultat aus dem Reusstal zeigt, dass sich die räumliche Isolation von Laubfroschbeständen tatsächlich negativ auf deren Lebensfähigkeit auswirken kann. Dieses Resultat unterstreicht zusätzlich die Bedeutsamkeit von Vernetzungsmassnahmen. Unsere genetischen Analysen zeigten, dass der isolierte Bestand bei Brugg keinerlei Individuenaustausch mit anderen Beständen aufweist. Die Rufchöre in diesem Bestand sind über die letzten Jahre zudem kleiner geworden, was auf eine verminderte Lebensfähigkeit der Laubfrösche in diesem Bestand hindeuten kann. Da die grosse räumliche Distanz zu den nächsten Beständen innerhalb nützlicher Frist kaum wirksame strukturelle Vernetzungsmassnahmen zulässt, wäre es hier sinnvoll, künstlich Individuen auszutauschen. Als Herkunftsort können Laubfrösche aus der Umgebung von Melligen und Bremgarten dienen, die aber zuvor auf Befall von Chytridiomykose untersucht werden müssen.

Basierend auf unseren Resultaten ist die Qualität der Landschaft zwischen Trittsteingewässern für die Laubfrösche eher sekundär, solange sich qualitativ hochwertige Trittsteingewässer im Umkreis von 2 km befinden. Ein funktionierendes Lebensraumnetzwerk für Laubfrösche muss also engmaschig sein, wobei räumliche Distanzen von über 4 km zwischen Teichen nicht überschritten werden dürfen. Die von der Praxis erfolgreich durchgeführten Massnahmen von Habitatsschutz, -aufwertung und -vernetzung sollten in Zukunft im Gesamtpaket weiterverfolgt werden, denn damit kann in Etappen eine funktionale landschaftsweite Vernetzung erreicht werden.

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Abbildung 1. Rufendes Laubfroschmännchen. Gut sichtbar ist die für jedes Individuum charakteristische Seitenlinie (Foto: Joggi Rieder-Schmid).

Abbildung 2. Frisch geschaffener Trittsteinweiher im Reusstal (Foto: Christoph Flory).

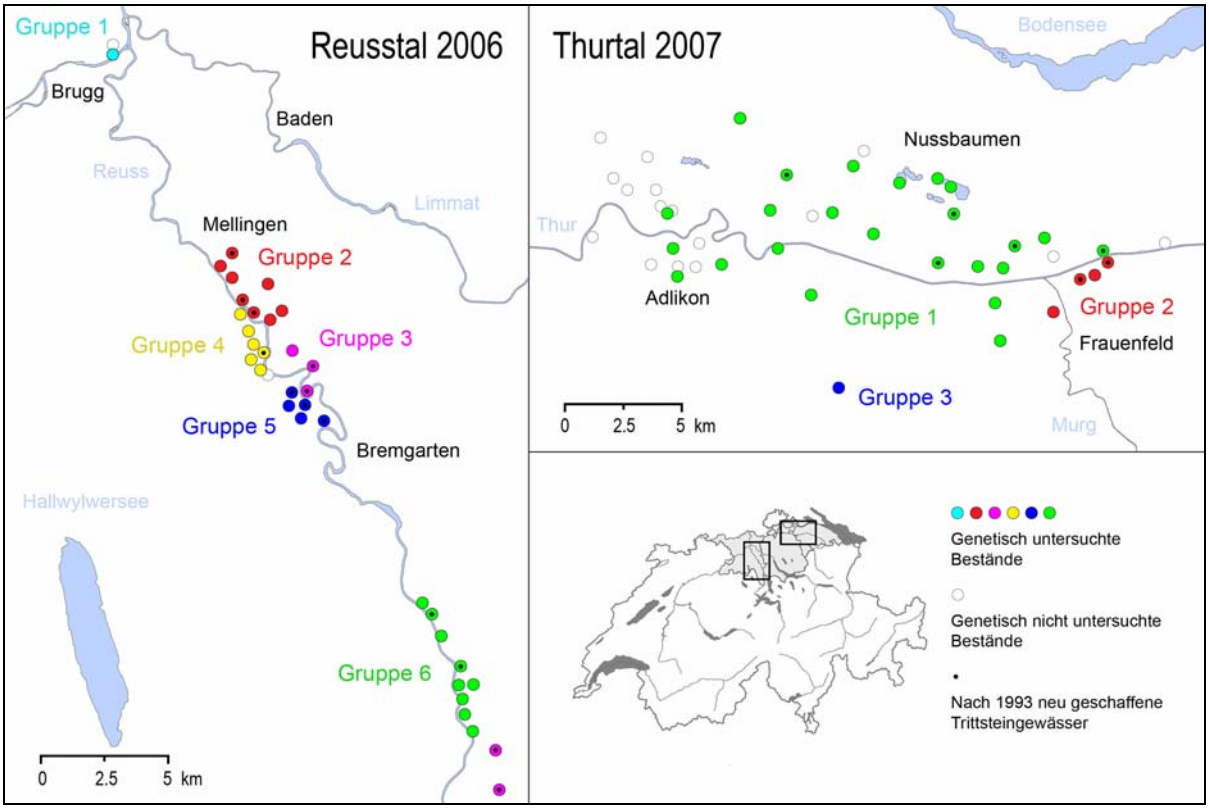

Abbildung 3. Einordnung der untersuchten Laubfroschbestände in genetische Gruppen im Reuss- und Thurtal.

SUMMARY

The preservation of species and habitats of conservation value is financially supported by many European countries. Hence, the Swiss Government also spends large sums of money on the implementation of connectivity measures. Their aim is to enhance the dispersal of individuals and gene flow among remnant populations within fragmented landscapes. These processes are essential for the long-term survival of endangered species as they counteract the negative effects of genetic erosion. Connectivity projects result from the creation of corridors, over- and underpasses across roads, or stepping-stones in between occupied habitat patches.

The evaluation of the effectiveness of connectivity measures is of great interest in conservation management. Does spatial connectivity actually translate into functional connectivity across landscapes? Answering this question raises a methodological problem because direct observations of animal movement or recapture of marked individuals are cost and labour intensive. Furthermore, corresponding results are often obtained at small special scales and their interpolation to the landscape level is difficult. Genetic methods, however, provide an alternative to ecological and demographic approaches to study individual movement and dispersal across a landscape.

The goal of this study was to use genetic methods to evaluate the effectiveness of connectivity projects on an endangered species and to test for fitness differences among its remnant populations. The situation of the European tree frog (*Hyla arborea* L.) in the Reuss and Thur river valleys in Switzerland offered an excellent study system to investigate the impact of connectivity measures in two independent landscapes differing in tree frog population density and levels of realised connectivity measures.

In **chapter 1**, the population history of tree frogs in the Reuss and Thur river valleys was assessed, the existent breeding sites were comprehensively sampled to determine their genetic structure based on eleven microsatellites, and first-generation migrant assignment tests were used to evaluate contemporary exchange of individuals among breeding sites. The analyses showed that the connectivity measures implemented in the Reuss valley afforded effective tree frog movement among breeding sites separated by distances of up to 4 km. In the Reuss valley, six spatial genetic clusters of breeding sites were explicitly defined and reflected the

effects of obstacles to tree frog movement at larger spatial scales. In contrast, a large number of breeding sites had been preserved in the Thur valley and therefore still exhibited substantial genetic admixture.

In **chapter 2**, a landscape genetic analysis of the Reuss valley was performed by exploring the effects of landscape elements and geographic distance on genetic differentiation of tree frog breeding sites. At distances below 2 km, the river Reuss acted as barrier to gene flow whereas surrounding tree frog breeding sites had a positive effect. At distances between 2 km and 8 km, geographic distance, wetlands, amphibian areas, roads and forests had all negative effects on gene flow and no landscape element with a positive effect was detected. At distances exceeding 8 km, the dispersal limit of tree frogs was probably reached. The results show that the effect of the landscape on tree frog movement was clearly scale-dependent.

In **chapter 3**, tree frog larvae from the Reuss valley were reared in a common garden experiment to investigate whether larvae from genetically different clusters differed in fitness traits. The fitness-related variables measured were growth rates, developmental rates and survival at five larval stages from eclosion to metamorphosis. Significant cluster differences in terms of lower growth rates at early larval stages were revealed in one of the three genetic clusters studied, which was genetically less diverse and spatially isolated. The observed differences were linked to a bottleneck that affected this cluster in the 1980s. Hence, it was likely that genetic load acted on the tree frogs from this particular cluster.

Chapter 4 represents a report for conservation practitioners, highlighting the most important findings of the study and containing management recommendations for future tree frog conservation. Responsible authorities are encouraged to continue pursuing measures enhancing both the quality of tree frog breeding sites and the connectivity among them, as the combination of these measures is successful. Since the results from both genetic and landscape analyses indicated that tree frogs did not overcome spatial gaps larger than 8 km and that they started to perceive movement costs at distances exceeding 2 km, a functional habitat network for European tree frogs in fragmented landscapes should have a maximum mesh width of 2 km.

ZUSAMMENFASSUNG

Die Erhaltung schutzwürdiger Arten und Lebensräume wird in vielen Europäischen Ländern mit staatlichen Mitteln unterstützt. So fördert auch die Schweiz Vernetzungsprojekte mit beträchtlichen Geldsummen. Dies hat zum Ziel, die Ausbreitung von Individuen und damit den Genaustausch zwischen Restpopulationen in zerschnittenen Landschaften zu erhalten. Vernetzung ist für das längerfristige Überleben bedrohter Arten wichtig, um der genetischen Verarmung und deren negativen Folgen entgegen zu wirken. Vernetzung erfolgt durch landschaftsgestalterische Massnahmen wie die Erstellung von Korridoren, Grünbrücken und Tunnels über oder unter Strassen, sowie von Trittsteinelementen zwischen bereits besetzten Habitatsflächen.

Erfolgskontrollen von Vernetzungsmassnahmen sind wichtig für den praktischen Naturschutz. Führt räumliche Vernetzung tatsächlich zu funktionaler, landschaftsweiter Vernetzung? Die Beantwortung dieser Frage ist für die Praxis problematisch, weil direkte Erfassungen von Tierbewegungen oder das Wiederfangen zuvor markierter Individuen schwierig durchzuführen und sehr Arbeitsintensiv sind. Zudem beschränken sich solche Untersuchungen häufig auf kleine Gebiete, was die Interpretation der Resultate auf landschaftsweiter Ebene erschwert. Im Gegensatz zu diesen Methoden bieten genetische Methoden eine geeignete Alternative, um die Bewegung und die Ausbreitung von Individuen in einer Landschaft zu erfassen.

Die vorliegende Studie überprüft die Wirksamkeit von Vernetzungsmassnahmen für eine bedrohte Art mit Hilfe genetischer Methoden und untersucht die restlichen Bestände auf Unterschiede in ihrer Lebensfähigkeit. Die Schweizer Vorkommen des Europäischen Laubfrosches (*Hyla arborea* L.) im Reuss- und Thurtal bieten ideale Studiensysteme, um den Einfluss von Vernetzungsmassnahmen in zwei unabhängigen Gebieten zu untersuchen, die sich in der Bestandesdichte des Laubfroschs, sowie im Umfang der getroffenen Vernetzungsmassnahmen unterscheiden.

In **Kapitel 1** wurde die Bestandesgeschichte des Laubfroschs in beiden Untersuchungsgebieten erfasst und die heutigen Vorkommen umfassend beprobt, um deren genetische Struktur anhand von elf Mikrosatelliten zu bestimmen. Der aktuelle Individuenaustausch zwischen den einzelnen Beständen wurde mit Zuordnungstests der genetischen Fingerabdrücke abgeschätzt (Erstgeneration-Migranten). Die Analysen zeigten, dass die Vernetzungsmassnahmen im Reusstal aktuellen Genfluss

zwischen Beständen im Umkreis von 4 km ermöglichten. Die Bestände waren in sechs geographisch umrissene genetische Gruppen unterteilt, was zeigt, dass gewisse Elemente in der Landschaft die Fortbewegung über grössere Distanzen hemmen. Im Thurtal hingegen ist der Hauptteil der Bestände erhalten geblieben und weist deshalb heute noch immer eine hohe genetische Durchmischung auf.

In **Kapitel 2** wurde in einer landschafts-genetischen Analyse die Auswirkung von Landschaftselementen und der geographischen Distanz auf den Genfluss im Reusstal untersucht. Es zeigte sich, dass zwischen Beständen in Abständen bis zu 2 km die Reuss eine Barriere für Genfluss bildete, während umliegende Laubfrosch-gewässer einen positiven Einfluss ausübten. In Abständen zwischen 2 km und 8 km übten die geographische Distanz, Feuchtgebiete, Amphibiengewässer, Strassen und Wälder einen negativen Einfluss auf den Genfluss aus. Abstände von über 8 km wurden von Laubfröschen kaum überwunden. Die Resultate zeigen, dass der Effekt der Landschaft auf den Genfluss eindeutig vom Distanzmass abhängig war.

In **Kapitel 3** wurde in einem Aufzuchtsexperiment analysiert, ob Laubfroschlarven aus drei verschiedenen genetischen Gruppen Unterschiede in ihrer Lebensfähigkeit und Entwicklung aufwiesen. Es wurden Wachstums-, Entwicklungs- und Überlebens-raten in fünf Stadien vom Schlüpfzeitpunkt der Larven bis zur Metamorphose berechnet. Dabei zeigten Larven aus einer genetischen Gruppen geringere Wachstumsraten in frühen Stadien. Die Bestände dieser Gruppe waren räumlich isoliert und wiesen eine reduzierte genetische Vielfalt auf, was beides von einem demographischen Flaschenhals herstammen könnte, der in den 1980er Jahren auf diese Bestände einwirkte. Es ist deshalb möglich, dass die Laubfrösche dieser Bestände von einer genetischen Bürde beeinträchtigt werden.

Kapitel 4 ist ein Umsetzungsartikel für die Naturschutzpraxis, der die Haupt-resultate der Studie vorstellt und Handlungsempfehlungen für zukünftige Schutz-massnahmen für den Laubfrosch enthält. Den zuständigen Behörden wird empfohlen, Massnahmen für die Habitatsaufwertung und Vernetzung weiter zu ver-folgen, denn die Kombination dieser Massnahmen ist erfolgreich. Da die genetischen Analysen zeigten, dass Distanzen über 8 km von Laubfröschen kaum überwunden werden und sich Ausbreitungskosten bereits ab 2 km bemerkbar machen, muss ein funktionales Netzwerk von Laichgewässern für den Europäischen Laubfrosch eine Maschenweite von maximal 2 km aufweisen.

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CURRICULUM VITAE

Personal

Name	Sonia ANGELONE
Birth	March 16th, 1975, in Männedorf ZH
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Education

2006 – 2009	PhD thesis at University of Zurich: 'Dispersal success of European tree frogs thanks to habitat connectivity measures: A genetic evaluation' supervised by Prof. Dr. Rolf Holderegger, WSL Swiss Federal Research Institute.
2001 – 2002	Diploma thesis at University of Zurich: 'Genetic variability across a vertebrate species' range: Comparisons of central and peripheral populations of *Rana latastei*' supervised by Dr. P.B. Pearman and Dr. T.W.J. Garner, University of Zurich.
1996 – 2000	Bachelor and master studies in Biology at the University of Zurich. Main subject: Zoology, and subsidiary subject: Anthropology.
1990 – 1995	Matura Typus D (with focus on French, Italian and English) at Kantonsschule Riesbach in Zurich.

Employment

2003 – 2006	Research associate in the Section Ecological Genetics, WSL Swiss Federal Research Institute: 'Promotion of rare tree species in Switzerland', a programme supported by the Swiss Federal Office for the Environment (BAFU)".

Nature conservation activities

1995 – 2000	Several volunteer stays at the Centro Méxicano de la Tortuga, Oaxaca, Mexico, and in the National park Lagunas de Chacahua, Oaxaca, Mexico, mainly working on the conservation of sea turtles at different beaches.

Activities during the PhD project

Elaboration and submission of the research proposal; acquisition of research funds (grants received by: MAVA Foundation, U. W. Linsi Stiftung, Wolfermann Nägeli Stiftung, Stiftung Seebachtal, Baudepartement Aargau, Amt für Landschaft und Natur Kanton Zürich, Amt für Raumplanung des Kantons Thurgau); oral and poster presentations in Switzerland and abroad; substantial outreach work (newspapers, radio); realization of a tree frog card game and co-production of a stand for the Natur 2007 exhibition in Basel; co-supervision of a master student and a postdoctoral fellow; reviews for ISI journals.

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